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THE TOXIC EFFECT OF CERTAIN CHEMICAL SOLUTIONS ON SPORES OF PENICILLIUM ITALICUM AND P. DIGITATUM[1,2]

LIANG HWANG[3] AND L. J. KLOTZ[4]

INTRODUCTION

THE BLUE AND THE GREEN molds (*Penicillium italicum* Wehmer and *P. digitatum* Sacc.) are the most common fungi causing soft decay in citrus fruits. They are world-wide in distribution, affecting fruits in orchards, in packing-houses, during transportation, and on the markets. In 1908, Powell (*27*)[5] reported that the losses from blue-mold decay in oranges during transportation from California were from $750,000 to $1,500,000 annually.

According to Sawada's (*34*) report in 1922, the two molds caused decay of oranges in Italy, the United States, Japan, and Formosa. Tindale (*38*) stated that in Victoria blue and green molds are the greatest enemies of oranges in cold storage and elsewhere. He (*39*) also reported that after two months' cold storage blue mold developed extensively. In 1928, Barker (*7*) stated that green mold causes serious losses in oranges from Spain, Palestine, Brazil, and the Argentine; less extensive damage is caused to oranges from South Africa, Australia, and California, and to grapefruit from Florida, Puerto Rico, and South Africa. In the same year Reichert and Littauer (*32*) reported that blue and green molds developed on picked fruit in Palestine. Bates (*8*) has shown that ship-

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[2] Paper No. 367, University of California Citrus Experiment Station and Graduate School of Tropical Agriculture, Riverside, California.

[3] Research Fellow of China Foundation for the Promotion of Education and Culture.

[4] Associate Plant Pathologist in the Experiment Station.

[5] Italic numbers in parentheses refer to "Literature Cited" at the end of this paper.

ment of early varieties in South Africa without precooling was attended by a very marked increase in mold wastage on discharge overseas. Yu (*43*) has reported that blue and green molds are found on all kinds of citrus fruits on the markets in China. Recently, Nattrass (*24*) reported that wastage of Cyprus oranges arriving in Europe was caused by blue and green molds.

In 1925, H. S. Fawcett (*13*) reported that of the decay of citrus fruits on arrival at eastern markets, 1.4 per cent was due to blue mold and 0.8 per cent was due to green mold. He also mentioned that the principal and almost the only kinds of decay found in California were the green mold and blue mold. In 1928, Barger (*4*) reported that Fawcett, after inspecting 500 field boxes of navel oranges in 8 Tulare County packing-houses in California in 1927, found that among the 1 per cent of rot in these oranges, 52 per cent of it was due to green mold, 32 per cent to a mixture of blue and green molds, 11 per cent to blue mold, and 5 per cent to other types of decay. According to Hopkins' report (*20*) for 1929, it was shown that the greatest losses during storage were due to *Penicillium italicum* and other fungi. Takeuchi (*37*) reported in 1929 that the rotting of satsuma oranges in storage or transit in Japan was caused by *P. italicum*, *P. digitatum*, and two other species of *Penicillium*. It was estimated that 89 per cent of the decayed fruits had *P. italicum* and 73 per cent had *P. digitatum*.

The prevention of any form of wounds is the most important means of reducing decay, but the use of solutions of certain substances has been tried out as a supplementary means to prevent the rot. A solution of borax was first tested and described by Fulton and Bowman (*17*) in 1924. Further suggestions and confirmations for its use have been reported by the following: Fulton and Winston (*18*), Barger and Hawkins (*6*), Barger (*4*), the Brogdex Company (*10*), Powell (*28*), Young and Read (*42*), Benton (*9*), Bates (*8*), Nattrass (*24*), Putterill (*30*), and by Winston (*41*). The solutions of soap and of borax used in citrus-fruit packing houses for washing and disinfecting have been reported by Fawcett (*14*) and by Shiver (*35*). Hodgson (*19*) claimed that the use of borax was rapidly going out of favor in the California citrus industry. Some workers (*8, 29*) have emphasized that borax treatment can be considered only as an adjunct to careful handling.

"Metbor," a new material said to equal borax as a decay preventive, was reported recently by Stewart (*36*) to have marked advantages over borax in regard to cold water solubility and other properties.

According to the Charter Oak House tests (*11*), the sodium hypochlorite process will prevent blue and green molds from developing in fruit

while it is in transit. Recently a stabilized sodium hypochlorite concentrate (*2*) has been manufactured; this contained 6 per cent sodium hypochlorite and when diluted according to directions was effective in controlling blue mold on apples and pears. Baker and Heald (*3*) found that rinsing apples for one minute with a sodium hypochlorite solution containing 0.4 per cent available chlorine was very effective in reducing the number of viable spores of *Penicillium expansum* on the surface and in the lenticels of apples and in decreasing losses from decay by this fungus.

Sodium bicarbonate and sodium carbonate are used in countries where borax treatment is prohibited by law. In 1928, Barger (*5*) first used sodium bicarbonate in controlling molds. The results were confirmed by Young and Read (*42*), by the Australian Citrus Preservation Committee (*1*), by Benton (*9*), by Bates (*8*), by Putterill and Davies (*31*), and by Putterill (*30*). Sodium carbonate was shown by Doidge (*12*) to be best for the control of *Penicillium* molds.

Tomkins and Trout (*40*) stated that storage of oranges in a humid atmosphere with ammonium carbonate or with crystals of ammonium carbonate reduces green-mold decay.

The study reported here was made for the purpose of securing more definite and effective means of controlling the blue and green molds by the use of chemical solutions.

MATERIALS USED

The original cultures of *Penicillium italicum* (No. 1746) and *P. digitatum* (No. 1438) were obtained from the stock cultures of the Division of Plant Pathology, Citrus Experiment Station, Riverside, California. The former was isolated by L. J. Klotz in 1930 from a decayed Valencia orange, and the latter by G. Savastano in 1927 from a decayed lemon. The medium used for these two fungi was 2 per cent glucose potato agar in the form of test-tube slants.

All cultures used throughout the experiments were incubated at 77° F (25° C), which was near the optimum temperature for growth on culture media as well as on orange fruits, as shown by Fawcett and Barger (*15*). The rate of sporulation of *Penicillium digitatum* is much slower than that of *P. italicum*. For the purpose of getting fair sporulations of these two fungi, *P. digitatum* was transferred to the slant 8 days earlier than *P. italicum*. For all treatments throughout the experiments, the age of *P. italicum* cultures used was 6 days and that of *P. digitatum* was 14 days.

The substances described below were used in solution form for treating both kinds of spores in the experiments:

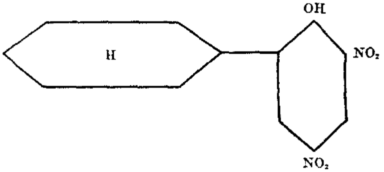
1. It was thought that a neutral soap of high purity might be used to facilitate wetting and to prevent clumping of spores. Accordingly a good grade of Castile soap was selected and tested in various concentrations.

2. Various concentrations of borax (sodium tetraborate decahydrate), $Na_2B_4O_7 \cdot 10H_2O$, were used. This preparation is also called sodium biborate or sodium pyroborate. It is a colorless monoclinic crystal or white powder and is slightly soluble in water and insoluble in alcohol.

3. A 6 per cent solution of a mixture of 2 parts borax and 1 part boric acid, H_3BO_3, was employed.

4. Metbor, which consists of 95 to 97 per cent sodium metaborate ($Na_2B_2O_4 \cdot H_2O$) and 3 to 5 per cent borax, was used in various concentrations.

5. Dinitro-o-cyclohexylphenol, is a yellow powder, having the empirical formula $C_{12}H_{14}N_2O_5$, and the structural formula:



It is only slightly soluble in distilled water, dissolving to the extent of 6.2 milligrams per liter of water. It was used in concentrations representing saturation, half saturation, and one-fourth saturation.

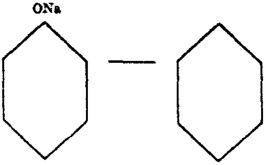
6. A 6 per cent sodium hypochlorite, NaOCl, was used as stock solution and then diluted to three concentrations of 1.0, 0.6, and 0.4 per cent.

7. Various concentrations of sodium bicarbonate, $NaHCO_3$, were used. This is also called acid sodium carbonate and baking soda. It is a white opaque powder or colorless crystals, soluble in water.

8. Sodium carbonate, Na_2CO_3 (anhydrous), was used in various concentrations. It is a white powder which is soluble in water. The form commonly used commercially is called soda ash.

9. A 0.4 per cent solution of chloramine-T or chlorazene, $CH_3C_6H_4SO_2Na:NCl \cdot 3H_2O$ (sodium p-toluene-sulfo-chloramine), was also tested. It is also called mianine, actirin, tochlorine, chloroamine, tolamine, and Dakin's antiseptic. It takes the form of colorless crystals which are soluble in water.

10. Sodium o-phenylphenate was used as a 0.15 per cent solution. The chemical is represented by the formula:



It is a white powder which is difficultly soluble in water.

11. A 1 per cent solution of a commercial washing powder containing mostly soda ash (anhydrous sodium carbonate) with some caustic and a trace of pine oil, was also used.

METHODS

The methods described here have been used throughout the experiments as the standard procedure, unless otherwise stated. The more specific methods will be described under separate headings.

As soon as preparations for a certain experiment were completed the spore suspensions were made up in 0.25 per cent soap solution from agar slant cultures of *Penicillium italicum* and *P. digitatum*. About 10 minutes after shaking these suspensions, 5 cc of each of the suspensions was transferred with sterile pipettes to sterile centrifuge tubes. In order to precipitate the spores from suspension, these tubes were centrifuged for 3 minutes. Immediately after that the supernatant solution was decanted and into each tube was poured 10 cc of a desired concentration of the designated chemical. The tube was then shaken thoroughly. About 3 minutes before the desired treatment time had expired, the tubes were put in the centrifuge in order to precipitate the treated spores. At the end of 3 minutes, the treating solution was decanted and the spores were washed with sterile distilled water.

When experiments on the effect of various temperatures were conducted, treating solutions at the desired temperature were poured on the spores and the tubes immersed immediately in water baths at definite, controlled temperatures for a period of about 3 minutes less than the desired period of exposure. Then these tubes were put in the centrifuge which was fixed in an electric oven adjusted to the same temperature as the water bath. After the tubes were centrifuged for 3 minutes, the treat-

ing solution was decanted and the spores washed with sterile distilled water as before. The check tubes were secured after the spore suspensions in soap were centrifuged and decanted, by using 10 cc of sterile distilled water instead of the treating solution. At the end of the treatments, the number of spores per cubic centimeter was estimated by means of a Howard counting chamber. Generally, two counts for each fungus were made, one of the treated suspension and one of the untreated suspension. In order to determine the viability of the treated and untreated spores, germination and dilution plate tests in triplicate were conducted as follows.

Germination.—For the sake of obtaining accurate results in spore-germination tests, several factors mentioned by McCallan and Wilcoxon (*23*) were considered. They stated that the most important factors are: cleanliness of glassware, source and age of spores, density of spore suspension, germination medium, concentration of toxic agent, temperature, and time. For the germinations two Van Tieghem cells were sealed to each glass slide with vaseline. A drop of sterile distilled water was placed in each cell and a small amount of vaseline on the upper edge of each ring. A very small drop of fresh sweet-orange juice and one loopful (4 mm) of spore suspension were placed on a sterile cover glass which was then inverted over a cell. Then these cells were placed in the incubator at 77° F (25° C). After 24 hours of incubation, a drop of chloroform was introduced into one cell of a slide to stop the growth during the period of measurement; this was repeated in the other cell after 48 hours.

Dilution Method.—As soon as the hanging-drop germination tests were completed, dilutions of 1:10,000, 1:100,000, and 1:1,000,000 were made by means of sterile pipettes, and 9 cc and 99 cc water blanks. Each of the dilutions was transferred with a pipette to a sterile petri dish. Melted glucose potato agar was poured into these petri dishes, and was mixed and incubated at 77° F. After periods of 2 days and 3 days, the number of colonies in the dishes was counted.

All results shown in table 2 were recorded as an average of three tests for each experiment, unless otherwise noted. The average number of colonies per cubic centimeter in the dilution-plate counts was calculated by dividing the total number of colonies of the three dilutions by the decimal 0.000111, since dilutions of 1:10,000 (0.0001), 1:100,000 (0.00001), and 1:1,000,000 (0.000001) were used. The viability index was calculated by dividing the average number of colonies per cubic centimeter of dilution-plate counts by the average number of spores per cubic centimeter of the microscopic counts. In the last column of table 2 the viability of each fungus after each treatment is calculated on the

basis of an assumed value of 100 for the viability in the water check. This permits comparison of the two fungi in any given solution and gives at a glance the relative efficiency of the several treatments.

MEANS OF WETTING SPORES

Before starting the experiments on the effect of various chemical solutions, three different experiments were made with soap, the object being to determine the most suitable concentration for wetting spores and preventing clumping.

The Effect of Various Concentrations of Soap Solution.—Spore suspensions were made in five different concentrations of Castile soap, 10

TABLE 1

CONCENTRATION OF SOAP SOLUTION IN RELATION TO THE NUMBER OF SPORE CLUMPS AT ROOM TEMPERATURE

Fungus	Treatment		Average number of clumps per field*	Number of spores per cubic centimeter
	Per cent soap	Time, in minutes		
Penicillium italicum	0.10	10	4.5	3,837,157
	0.25	10	6.7	
	0.50	10	5.9	
	1.00	10	6.1	
	2.00	10	6.5	
	Check	..	9.6	
Penicillium digitatum	0.10	10	7.4	2,[illegible]
	0.25	10	3.1	
	0.50	10	4.8	
	1.00	10	6.5	
	2.00	10	3.4	
	Check	..	3.8	

* Average of 20 fields under the low power of the microscope.

cc of each being placed in sterile centrifuge tubes. These were centrifuged at room temperature for about 3 minutes, the total length of exposure being 5 minutes. Immediately after centrifuging, all the soap solutions were decanted and the tubes filled with 10 cc of sterile distilled water. The germination tests and plate method were then carried out as described above. The results are shown in table 2, entries 1 to 12.

Although none of these concentrations showed much inhibition of spore germination, the results (table 2) indicated that 0.25 per cent soap solution had the least effect on both spore germination and growth in petri dishes in the case of *Penicillium digitatum.*

TABLE 2

Toxicity of Various Chemical Solutions to the Conidia at Several Temperatures and Periods of Exposure

Entry No.	Fungus	Fungicide	Concentration of fungicide, per cent	pH of fungicide	Temperature during treatment, °F	Exposure, minutes	Average per cent germination: After 24 hrs.	Average per cent germination: After 48 hrs.	Average number of colonies per cc by dilution plate counts A	Average number of spores per cc by microscopic counts B	Viability index† A ÷ B	Relative viability (water at 69° F = 100)
1			0.10	9.73	69	5	98.9	100.0	2,801,901	3,950,614	0.709	77.3
2			0.25	9.90	69	5	99.5	100.0	3,108,108	3,950,614	0.787	85.8
3		Castile soap	0.50	9.88	69	5	99.1	100.0	3,576,576	3,950,614	0.905	98.6
4	*P. italicum*		1.00	9.97	69	5	98.6	100.0	4,279,279‡	3,950,614	1.083	118.1
5			2.00	10.05	69	5	99.5	100.0	5,189,189‡	3,950,614	1.313	143.1
6		Distilled water	Check	4.98	69	5	99.5	100.0	4,189,189	4,567,898	0.917	100.0
7			0.10	9.73	69	5	68.8	92.5	1,522,522	2,304,525	0.661	90.3
8			0.25	9.90	69	5	76.9	98.7	1,531,531	2,304,525	0.664	90.7
9		Castile soap	0.50	9.88	69	5	70.8	84.0	1,423,423	2,304,525	0.618	84.4
10	*P. digitatum*		1.00	9.97	69	5	72.8	100.0	1,270,270	2,304,525	0.551	75.2
11			2.00	10.05	69	5	71.5	82.5	1,324,324	2,304,525	0.575	78.5
12		Distilled water	Check	4.98	69	5	76.6	92.5	1,837,837	2,510,289	0.732	100.0
13			0.10; 6¶	9.30	69	10; 5¶	52.2	93.4	2,909,909	3,497,939	0.832	71.5
14			0.25; 6	9.30	69	10; 5	46.6	99.2	2,765,765	3,497,939	0.791	68.0
15		Castile soap; borax	0.50; 6	9.30	69	10; 5	29.1	91.9	3,090,090	3,497,939	0.883	75.9
16			1.00; 6	9.30	69	10; 5	32.3	75.8	4,189,189‡	3,497,939	1.198	103.0
17	*P. italicum*		2.00; 6	9.30	69	10; 5	31.8	96.4	4,171,171‡	3,497,939	1.192	102.5
18			0.00; 6	9.30	69	5	41.4	98.7	3,090,090	3,497,939	0.883	75.9
19		Distilled water	Check	4.98	69	5	96.2	100.0	5,981,981‡	5,144,022	1.163	100.0
20			0.10; 6	9.30	69	10; 5	18.8	28.1	1,000,000	2,716,047	0.368	62.6
21			0.25; 6	9.30	69	10; 5	13.8	54.3	846,846	2,716,047	0.312	53.1
22		Castile soap; borax	0.50; 6	9.30	69	10; 5	4.1	36.5	936,936	2,716,047	0.345	58.7
23			1.00; 6	9.30	69	10; 5	22.7	75.5	684,684	2,716,047	0.252	42.9
24	*P. digitatum*		2.00; 6	9.30	69	10; 5	43.6	74.9	990,990	2,716,047	0.365	62.1
25			0.00; 6	9.30	69	5	59.2	93.6	1,045,045	2,716,047	0.385	65.5
26		Distilled water	Check	4.98	69	5	97.7	100.0	1,765,765	3,004,113	0.588	100.0

27	*P. italicum*	Distilled water	•	4.98	69	5	99.6	100.0	5,104,828	5,226,333	0.977	100.0
28				5.04	100	5	97.2	100.0	1,846,846	4,444,107	0.415	42.5
29				5.06	110	5	58.6	98.4	747,747	4,444,107	0.168	17.2
30				5.30	120	5	0.8	11.5	9,009	4,444,107	0.002	0.2
31	*P. digitatum*	Distilled water		4.98	69	5	99.2	100.0	1,504,504	3,168,721	0.475	100.0
32				5.04	100	5	95.8	99.6	792,792	2,921,908	0.271	57.1
33				5.06	110	5	59.2	99.6	509,509	2,921,908	0.174	36.6
34				5.30	120	5	0.4	8.2	9,009	2,921,908	0.003	0.6
35	*P. italicum*	Borax	6	8.98	110	2	65.5	100.0	3,666,666	8,768,759	0.418	88.4
36			6	8.98	110	4	80.0	100.0	2,882,882	8,768,759	0.329	69.6
37			6	8.98	110	6	13.6	81.6	2,774,774	8,768,759	0.316	66.8
38			6	8.98	110	8	13.1	57.0	1,549,549	8,768,759	0.177	37.4
39			6	8.98	110	10	1.2	24.4	531,531	8,768,759	0.061	12.9
40			6	8.98	110	12	2.4	39.9	792,792	8,768,759	0.090	19.0
41			6	8.98	110	14	0.1	24.3	441,441	8,768,759	0.050	10.6
42			6	8.98	110	16	0.1	22.1	230,630	8,768,759	0.026	5.5
43		Distilled water	Check	4.98	69	5	100.0	100.0	4,441,441	9,396,042	0.473	100.0
44	*P. digitatum*	Borax	6	8.98	110	2	68.1	99.1	1,171,171	3,580,177	0.327	60.8
45			6	8.98	110	4	82.6	99.4	837,837	3,580,177	0.234	43.8
46			6	8.98	110	6	32.1	64.2	884,884	3,580,177	0.247	45.9
47			6	8.98	110	8	3.6	12.4	504,504	3,580,177	0.141	26.4
48			6	8.98	110	10	3.9	33.4	301,901	3,580,177	0.084	15.7
49			6	8.98	110	12	3.6	27.9	141,141	3,580,177	0.039	7.3
50			6	8.98	110	14	1.0	5.5	193,693	3,580,177	0.054	10.0
51			6	8.98	110	16	0.9	4.9	128,928	3,580,177	0.036	6.7
52		Distilled water	Check	4.98	69	5	99.8	100.0	2,306,306	4,283,165	0.538	100.0

* *Total number of colonies* in three dilution plates *divided by* 0.000111.

† Average number of colonies per cubic centimeter determined by dilution plate count *divided by* the average number of spores per cubic centimeter determined by microscopic count *equals* the viability index.

‡ Theoretically, this figure should not have exceeded the microscopic count.

¶ In this column in entries 13 to 26, the first number refers to castile soap and the number following the semicolon refers to borax.

TABLE 2—(*Continued*)

Entry No.	Fungus	Fungicide	Concentration of fungicide, per cent	pH of fungicide	Temperature during treatment, °F	Exposure, minutes	Average per cent germination: After 24 hrs.	Average per cent germination: After 48 hrs.	Average number of colonies per cc by dilution plate counts* A	Average number of spores per cc by microscopic counts B	Viability index† A÷B	Relative viability (water at 69° F = 100)
53	*P. italicum*	Borax	6	9.30	69	5	49.0	78.6	3,162,162	4,691,354	0.674	62.1
54			6	9.14	80	5	40.9	73.2	4,109,106	4,691,354	0.876	80.7
55			6	9.00	100	5	36.5	40.9	3,261,261	4,691,354	0.695	64.0
56			6	8.96	110	5	12.9	40.8	1,567,567	4,691,354	0.334	30.8
57			6	8.93	120	5	0.1	2.3	0	4,691,354	0.000	0.0
58		Distilled water	Check	4.98	69	5	100.0	100.0	5,720,720‡	5,267,419	1.086	100.0
59	*P. digitatum*	Borax	6	9.30	69	5	51.5	77.1	1,207,207	3,786,006	0.319	56.8
60			6	9.14	80	5	55.7	62.3	1,297,297	3,786,006	0.343	61.0
61			6	9.00	100	5	39.7	58.2	1,027,027	3,786,006	0.271	48.2
62			6	8.96	110	5	5.2	53.5	585,585	3,786,006	0.155	27.6
63			6	8.93	120	5	1.0	1.3	2,702	3,786,006	0.001	0.1
64		Distilled water	Check	4.98	69	5	99.0	100.0	2,450,450	4,362,138	0.562	100.0
65	*P. italicum*	Borax	4	8.95	110	5	13.7	85.9	1,729,729	5,843,620	0.296	31.1
66			6	8.98	110	5	6.0	41.6	1,324,324	5,843,620	0.227	23.9
67			8	9.03	110	5	0.8	23.7	1,387,387	5,843,620	0.237	24.9
68			10	9.08	110	5	0.6	7.9	1,144,144	5,843,620	0.196	20.6
69			12	9.11	110	5	0.0	1.7	936,936	5,843,620	0.160	16.8
70		Distilled water	Check	4.98	69	5	100.0	100.0	6,621,621	6,962,546	0.951	100.0
71	*P. digitatum*	Borax	4	8.95	110	5	59.4	89.2	1,297,297	4,444,441	0.292	76.2
72			6	8.98	110	5	5.7	51.0	1,045,045	4,444,441	0.235	61.4
73			8	9.03	110	5	4.1	39.4	909,909	4,444,441	0.205	53.5
74			10	9.08	110	5	2.2	31.9	711,711	4,444,441	0.160	41.8
75			12	9.11	110	5	0.0	1.2	401,801	4,444,441	0.090	23.5
76		Distilled water	Check	4.98	69	5	100.0	100.0	1,891,891	4,938,938	0.383	100.0

77	P. italicum	Borax-boric acid	6	8.18	100	5	59.6	99.6	2,342,342	4,067,771	0.576	56.7
78			6	8.16	110	5	0.0	40.8	743,243	4,067,771	0.183	18.0
79			6	8.14	120	5	0.0	11.9	7,207	4,067,771	0.002	0.2
80		Distilled water	Check	4.98	69	5	100.0	100.0	5,225,225‡	5,144,029	1.016	100.0
81	P. digitatum	Borax-boric acid	6	8.18	100	5	90.8	100.0	1,153,153	2,757,199	0.418	94.1
82			6	8.15	110	5	4.6	89.9	495,495	2,757,199	0.176	39.8
83			6	8.14	120	5	0.0	0.0	2,702	2,757,199	0.001	0.2
84		Distilled water	Check	4.98	69	5	99.9	100.0	1,369,369	3,086,064	0.444	100.0
85	P. italicum	Metbor	6	10.18	69	2	99.2	100.0	2,261,261	3,621,396	0.624	61.7
86			6	10.18	69	5	91.8	100.0	2,216,216	3,621,396	0.612	60.5
87			6	10.18	69	10	87.7	99.3	1,882,882	3,621,396	0.520	51.4
88			6	10.18	69	15	65.6	91.7	1,756,756	3,621,396	0.485	48.0
89		Distilled water	Check	4.98		5	99.6	100.0	4,036,036‡	3,991,432	1.011	100.0
90	P. digitatum	Metbor	6	10.18	69	2	97.4	100.0	1,261,261	3,004,112	0.420	89.9
91			6	10.18	69	5	96.9	100.0	1,099,099	3,004,112	0.366	78.4
92			6	10.18	69	10	77.6	98.4	981,981	3,004,112	0.327	70.0
93			6	10.18	69	15	49.6	79.2	900,900	3,004,112	0.300	64.2
94		Distilled water	Check	4.98	69	5	99.9	100.0	1,576,576	3,374,482	0.467	100.0
95	P. italicum	Metbor	6	10.18	69	5	91.8	100.0	2,216,216	3,621,396	0.612	62.6
96			6	9.95	100	5	67.1	99.8	2,198,198	4,444,107	0.494	50.7
97			6	9.86	110	5	73.1	99.2	1,981,981	4,444,107	0.446	45.6
98			6	9.79	120	5	0.0	0.6	23,423	4,444,107	0.005	0.5
99		Distilled water	Check	4.98	69	5	99.6	100.0	5,108,108	5,226,333	0.977	100.0
100	P. digitatum	Metbor	6	10.18	69	5	96.9	100.0	1,000,000	3,004,112	0.333	70.1
101			6	9.95	100	5	59.5	95.8	639,639	2,921,806	0.219	46.1
102			6	9.86	110	5	36.3	91.0	630,630	2,921,806	0.216	45.5
103			6	9.79	120	5	0.0	0.4	2,702	2,921,806	0.001	0.2
104		Distilled water	Check	4.98	69	5	98.3	100.0	1,504,504	3,166,731	0.475	100.0

* Total number of colonies in three dilution plates *divided by* 0.000111.

† Average number of colonies per cubic centimeter determined by dilution plate count *divided by* the average number *of spores per cubic centimeter determined by* microscopic count *equals* the viability index.

‡ Theoretically, this figure should not have exceeded the microscopic count.

TABLE 2—(*Continued*)

Entry No.	Fungus	Fungicide	Concentration of fungicide, per cent	pH of fungicide	Temperature during treatment, °F	Exposure, minutes	Average per cent germination After 24 hrs.	Average per cent germination After 48 hrs.	Average number of colonies per cc by dilution plate counts* A	Average number of spores per cc by microscopic counts B	Viability index† A ÷ B	Relative viability (water at 69° F = 100)
105	*P. italicum*	*Methor*	4	10.07	69	5	78.0	94.5	2,171,171	3,621,396	0.599	59.2
106			6	10.15	69	5	91.8	100.0	2,216,216	3,621,396	0.612	60.5
107			8	10.22	69	5	73.2	91.2	1,909,909	3,621,396	0.527	52.1
108			10	10.27	69	5	44.3	76.8	1,900,900	3,621,396	0.525	51.9
109			12	10.31	69	5	31.9	60.2	1,864,864	3,621,396	0.515	50.9
110		Distilled water	Check	4.98	69	5	99.6	100.0	4,036,036‡	3,991,432	1.011	100.0
111	*P. digitatum*	Methor	4	10.07	69	5	97.8	99.0	1,198,198	3,004,112	0.399	85.4
112			6	10.18	69	5	96.9	100.0	1,099,099	3,004,112	0.366	78.4
113			8	10.22	69	5	78.2	92.5	918,918	3,004,112	0.306	65.5
114			10	10.27	69	5	56.5	83.8	576,576	3,004,112	0.192	41.1
115			12	10.31	69	5	35.4	65.4	441,441	3,004,112	0.147	31.5
116		Distilled water	Check	4.98	69	5	99.6	100.0	1,576,576	3,374,482	0.467	100.0
117	*P. italicum*	*Dinitro-o-*cyclohexylphenol	0.001	5.35	69	2	92.2	99.6	1,189,189	2,551,438	0.466	47.6
118			0.0005	5.16	69	2	91.5	100.0	1,162,162	2,551,438	0.455	46.5
119			0.00025	4.96	69	2	91.7	100.0	954,954	2,551,438	0.374	38.2
120		Distilled water	Check	4.98	69	5	99.5	100.0	5,117,117	5,226,333	0.979	100.0
121	*P. digitatum*	Dinitro-o-cyclohexylphenol	0.001	5.35	69	2	88.0	100.0	864,864	2,181,068	0.396	88.2
122			0.0005	5.16	69	2	94.4	100.0	1,018,018	2,181,068	0.466	103.8
123			0.00025	4.96	69	2	95.0	100.0	1,063,063	2,181,068	0.487	108.5
124		Distilled water	Check	4.98	69	5	99.7	100.0	1,459,459	3,251,026	0.449	100.0
125	*P. italicum*	NaOCl	0.4	11.10	69	2	0.0	0.0	0	7,736,619	0.000	0.0
126			0.6	11.14	69	2	0.8	2.0	0	7,736,619	0.000	0.0
127			1.0	11.14	69	2	0.0	0.0	0	7,736,619	0.000	0.0
128		Distilled water	Check	4.98	69	5	99.4	100.0	6,369,369	11,275,711	0.565	100.0

129		NaOCl	0.4 *	11.10	69	2	0.0	0.0	0	2,716,047	0.000	0.0
130			0.6	11.14	69	2	0.0	0.0	0	2,716,047	0.000	0.0
131	*P. digitatum*		1.0	11.14	69	2	0.0	0.0	0	2,716,047	0.000	0.0
132		Distilled water	Check	4.98	69	5	99.0	100.0	1,387,387	3,415,635	0.406	100.0
133		NaHCO$_3$	6	7.96	86	2	99.8	100.0	4,801,801	5,802,465	0.827	73.8
134			6	7.96	86	5	99.7	100.0	4,657,657	5,802,465	0.803	71.2
135	*P. italicum*		6	7.96	86	10	99.6	100.0	4,459,459	5,802,465	0.768	68.1
136		Distilled water	Check	4.98	69	5	99.7	100.0	7,522,522‡	6,666,661	1.128	100.0
137		NaHCO$_3$	6	7.96	86	2	99.7	100.0	1,171,171	3,086,417	0.379	62.9
138			6	7.96	86	5	99.6	100.0	1,261,261	3,086,417	0.409	67.8
139	*P. digitatum*		6	7.96	86	10	97.4	100.0	1,072,072	3,086,417	0.347	57.5
140		Distilled water	Check	4.98	69	5	99.8	100.0	2,009,009	3,333,330	0.603	100.0
141		NaHCO$_3$	6	7.96	86	5	99.7	100.0	4,657,657	5,802,465	0.803	69.2
142			6	7.85	*100*	5	99.2	*100.0*	*2,540,540*	4,444,437	0.572	49.3
143			6	7.85	110	5	96.9	100.0	2,162,162	4,444,437	0.486	41.9
144			6	7.82	120	5	0.0	25.2	18,018	4,444,437	0.004	0.3
145	*P. italicum*	Na$_2$CO$_3$	6	10.16	86	5	48.3	98.1	747,747	4,067,771	0.184	15.8
146			6	9.83	100	5	39.4	93.9	365,765	4,444,437	0.082	7.1
147			6	9.74	110	5	1.4	33.3	28,828	4,444,437	0.006	0.5
148			6	9.64	120	5	0.0	0.0	0	4,444,437	0.000	0.0
149		Distilled water	Check	4.98	69	5	100.0	100.0	5,540,540‡	4,773,659	1.161	100.0
150		NaHCO$_3$	6	7.96	86	5	99.8	100.0	1,261,261	3,086,417	0.409	78.4
151			6	7.85	100	5	99.2	100.0	1,351,351	2,921,808	0.462	88.5
152			6	7.85	*110*	5	98.5	*100.0*	*1,153,153*	*2,921,808*	*0.395*	75.7
153			6	7.82	120	5	0.5	11.8	23,423	2,921,808	0.008	1.5
154	*P. digitatum*	Na$_2$CO$_3$	6	10.16	86	5	61.5	91.8	459,459	2,767,199	0.166	31.8
155			6	9.83	100	5	44.7	78.0	450,450	2,921,808	0.154	29.5
156			6	9.74	110	5	0.4	6.0	28,828	2,921,808	0.010	1.9
157			6	9.64	120	5	0.0	0.0	0	2,921,808	0.000	0.0
158		Distilled water	Check	4.98	69	5	99.6	100.0	1,738,738	3,333,330	0.522	100.0

* Total number of colonies in three dilution plates *divided by* 0.000111.

† Average number of colonies per cubic centimeter determined by dilution plate count *divided by* the average number of spores per cubic centimeter determined by microscopic count *equals* the viability index.

‡ Theoretically, this figure should not have exceeded the *microscopic count*.

TABLE 2—(*Concluded*)

Entry No.	Fungus	Fungicide	Concentration of fungicide, *per cent*	pH of fungicide	Temperature during treatment, °F	Exposure, minutes	Average per cent germination: After 24 hrs.	Average per cent germination: After 48 hrs.	Average number of colonies per cc by dilution plate counts* A	Average number of spores per cc by microscopic counts B	Viability index† A ÷ B	Relative viability (water at 69° F = 100)
159	*P. italicum*	$NaHCO_3$	2	8.30	86	5	99.6	100.0	5,243,243	5,802,465	0.904	92.4
160			6	8.13	86	5	99.7	100.0	4,657,657	5,802,465	0.803	82.1
161			10	8.00	86	5	100.0	100.0	4,657,657	5,802,465	0.803	82.1
162		Distilled water	Check	4.98	69	5	99.7	100.0	6,522,522	6,666,661	0.978	100.0
163	*P. digitatum*	$NaHCO_3$	2	8.30	86	5	99.1	100.0	1,729,729	3,086,417	0.560	92.9
164			6	8.13	86	5	99.8	100.0	1,261,261	3,086,417	0.409	67.8
165			10	8.00	86	5	85.1	100.0	1,189,189	3,086,417	0.385	63.8
166		Distilled water	Check	4.98	69	5	99.8	100.0	2,009,009	3,333,330	0.603	100.0
167	*P. italicum*	Na_2CO_3	6	10.16	86	2	94.6	100.0	810,810	4,067,771	0.199	19.6
168			6	10.16	86	5	48.3	98.1	747,747	4,067,771	0.184	18.1
169			6	10.16	86	10	32.4	33.5	558,558	4,067,771	0.137	13.5
170		Distilled water	Check	4.98	69	5	100.0	100.0	5,225,225‡	5,144,029	1.016	100.0
171	*P. digitatum*	Na_2CO_3	6	10.16	86	2	70.7	100.0	657,657	2,757,199	0.238	53.6
172			6	10.16	86	5	61.5	91.8	459,459	2,757,199	0.167	37.6
173			6	10.16	86	10	30.9	63.4	324,324	2,757,199	0.118	26.6
174		Distilled water	Check	4.98	69	5	99.9	100.0	1,369,369	3,086,084	0.444	100.0
175	*P. italicum*	Na_2CO_3	2	10.20	86	5	99.0	100.0	2,837,837	4,067,771	0.698	68.7
176			6	10.15	86	5	48.3	98.1	747,747	4,067,771	0.184	18.1
177			10	9.90	86	5	39.4	72.9	309,903	4,067,771	0.083	8.1
178		Distilled water	Check	4.98	69	5	100.0	100.0	5,225,225‡	5,144,029	1.016	100.0
179	*P. digitatum*	Na_2CO_3	2	10.20	86	5	99.4	100.0	1,036,036	2,757,199	0.376	84.7
180			6	10.15	86	5	61.5	91.8	459,459	2,757,199	0.167	37.6
181			10	9.90	86	5	28.3	64.9	243,243	2,757,199	0.088	19.8
182		Distilled water	Check	4.98	69	5	68.9	100.0	1,369,369	3,086,084	0.444	100.0

183		Chloramine-T	0.4 *	6.52	69	5	87.1	98.5	873,873	4,444,107	0.197	24.5
184			0.4	6.47	100	5	94.4	100.0	711,711	2,592,590	0.274	34.1
185	*P. italicum*		0.4	6.45	110	5	0.0	78.5	9,009	2,592,590	0.003	0.4
186			0.4	6.55	120	5	0.0	0.0	0	2,592,590	0.000	0.0
187		Distilled water	Check	4.98	69	5	99.2	100.0	2,774,774	3,458,787	0.803	100.0
188		Chloramine-T	0.4	6.52	69	5	97.9	100.0	1,108,108	2,921,808	0.379	92.0
189			0.4	6.47	100	5	97.6	100.0	1,045,045	2,716,041	0.385	93.4
190	*P. digitatum*		0.4	6.45	110	5	20.8	97.1	234,234	2,716,041	0.086	20.9
191			0.4	6.55	120	5	0.0	0.0	0	2,716,041	0.000	0.0
192		Distilled water	Check	4.98	69	5	99.2	100.0	1,270,270	3,086,417	0.412	100.0
193		Sod. o-phenylphenate	0.15	9.67	69	5	42.4	98.6	1,468,468	4,444,107	0.330	33.8
194	*P. italicum*	Chloramine-T	0.40	6.52	69	5	87.1	98.5	873,873	4,444,107	0.197	20.3
195		Washing powder	1.00	9.90	69	5	98.1	100.0	2,648,648	4,444,107	0.596	61.0
196		Distilled water	Check	4.98	69	5	99.6	100.0	5,108,108	5,226,333	0.977	100.0
197		Sod. o-phenylphenate	0.15	9.67	69	5	5.2	63.6	128,828	2,921,808	0.044	9.3
198	*P. digitatum*	Chloramine-T	0.40	6.52	69	5	97.9	100.0	1,108,108	2,921,808	0.379	79.8
199		Washing powder	1.00	9.90	69	5	98.5	100.0	1,153,153	2,921,808	0.395	83.2
200		Distilled water	Check	4.98	69	5	99.2	100.0	1,504,504	3,168,721	0.475	100.0

* Total number of colonies in three dilution plates *divided by* 0.000111.

† Average number of colonies per cubic centimeter determined by dilution plate count *divided by* the average number of spores per cubic centimeter determined by microscopic count *equals* the viability index.

‡ Theoretically, this figure should not have exceeded the microscopic count.

The Concentrations of Soap Solution in Relation to Number of Spore Clumps.—Five concentrations of Castile soap solution were used to determine the best concentration for dispersing spores of the two molds. Spore suspensions were made with sterile, distilled water; each of 6 centrifuge tubes was then filled with 5 cc of the suspension. After centrifuging for 3 minutes, the supernatant water was decanted and the tubes were filled, respectively, with 10 cc of the above five concentrations of soap solution; the check tubes were filled with sterile, distilled water. Ten minutes after the solutions had been added, the spore clumps were counted by means of a Howard counting chamber under the low power of the microscope. The results are shown in table 1 (p. 7).

Table 1 shows that 0.25 per cent soap solution is the best concentration for dispersing spores of *Penicillium digitatum*, that is, fewer clumps appeared when this concentration was used. This is in harmony with the results on the effect of various concentrations of soap solution on spore germination and on the growth in petri dishes. In the case of *P. italicum* the fewest clumps appeared in the 0.10 per cent solution. Because of the indications of these experiments and because the several soap concentrations were all relatively nontoxic, a concentration of 0.25 per cent was chosen for use with both fungi in all experiments subsequent to the third (entries 35 *et seq.* of table 2).

Killing Effect of Borax in Relation to Concentration of Soap Solution.—In comparing the effect of the concentration of soap solution on the killing effect of borax, the following procedures were followed: First, treatment was made with different concentrations of soap solution for 10 minutes. This was followed by immersion in a 6 per cent borax solution for 5 minutes at room temperature (66° to 72°, average 69° F). Secondly, treatment was made with 6 per cent borax solution but without any previous treatment with soap solution. Finally, for checks, the spores were not subjected to borax treatment. Results are given in table 2, entries 13 to 26.

According to the results, a 6 per cent borax solution is more effective in checking the spore germination and the plate growth of both fungi when used in conjunction with concentrations of 0.1 to 1.0 per cent soap solutions.

From the results of the three experiments discussed above, it is also seen that 0.25 per cent soap solution is suitable for wetting spores of these fungi. Hereafter, unless otherwise noted, the spores for all the experiments were first wetted with this concentration of soap before any further tests were made.

EFFECT OF TEMPERATURE ON SPORES IN DISTILLED WATER

Experiments were conducted to find how different temperatures affected the spores of the two molds. Winston (*41*) had reported that water at 110° F or above showed effective control of decay.

The general procedure for this experiment was the same as stated above, except that special attention was paid to obtaining and maintaining the desired temperatures. The more important steps for this experiment may be described briefly as follows: When the spore suspensions were centrifuged and the supernatant soap solution was poured off, distilled water which had been previously heated to the desired temperature was poured into the centrifuge tube and shaken thoroughly. Immediately after that, the tubes were put in the water bath for about 2 minutes and then into the centrifuge, which had been placed in an electric oven at the same temperature. After having been centrifuged for about 3 minutes, the tubes were removed and the warm water replaced with sterile distilled water at room temperature. Germination and plating tests were then made; the results are reported in table 2, entries 27 to 34.

The data show that the percentage of germination of the spores as measured by direct counts or plate cultures decreases as the temperatures increase. At 120° F, germination and plate growth are greatly repressed.

EFFECT OF BORAX

The preliminary results of the experiments of Fulton and Bowman (*17*) have shown that a commercial borax solution of 5 per cent or 10 per cent greatly reduces blue-mold rot of citrus fruits under experimental conditions. Further experiments by Fulton and Winston (*18*) suggest the use of the 5 per cent borax solution at 120° F for 5 minutes. This procedure was later supported and patented by the Brogdex Company (*10*). The experiments by Barger and Hawkins (*6*) at first indicated that 2.5 per cent boric acid at 120° F gave very promising results in controlling blue mold (*Penicillium italicum*). Later borax was tested and found to be as effective as boric acid and was much cheaper than the latter. From the results based on experimental data and data obtained from the commercial shipments, Barger (*4*) concluded that both blue- or green-mold decay can be controlled by 7 per cent borax solution at 110° F for 5 minutes. This has been confirmed by Reichert and Littauer (*33*). As a result of experiments in South Africa, Powell (*28*) suggests the use of a hot 2.5

per cent borax solution, or boric acid, or mixtures of both for the control of the green mold (*P. digitatum*). Benton (*9*) found that a 4-minute immersion in 8 per cent borax solution at 110° F was effective in preventing decay in oranges. By the results of tests, Putterill (*30*) supports the use of 8 per cent borax. He also mentions that 4 per cent borax and 4 per

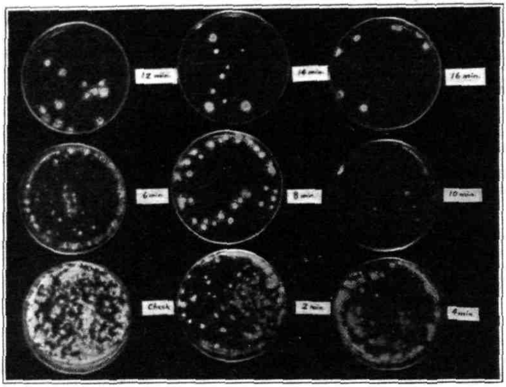


Fig. 1.—Effect on spores of *Penicillium italicum* exposed to 6 per cent borax solution at 110° F for various lengths of time. The plates represent a 1:100,000 dilution of the spore suspensions. The untreated suspension yielded numerous colonies, while treatments of 10 minutes or longer killed most of the spores.

cent sodium bicarbonate are of equal effectiveness at high temperatures, but the former is less effective at lower temperatures.

In order to determine the effect of the interrelation of concentrations of borax solution, with the length of treatment and various temperatures on the spores of both *Penicillium italicum* and *P. digitatum*, the following three experiments were conducted.

Time of Exposure.—In each series of 16 tests, 6 per cent borax solution at 110° F (43° C) was used for 2, 4, 6, 8, 10, 12, 14, and 16 minutes; as checks, 2 of the tests were made without chemical treatment except in 0.25 per cent soap solution. The results are given in table 2, entries 35 to 52, and shown in figure 1.

The results show that the percentage of spore germination of *Penicillium italicum* in 6 per cent borax at 110° F for 16 minutes is 0.1, but

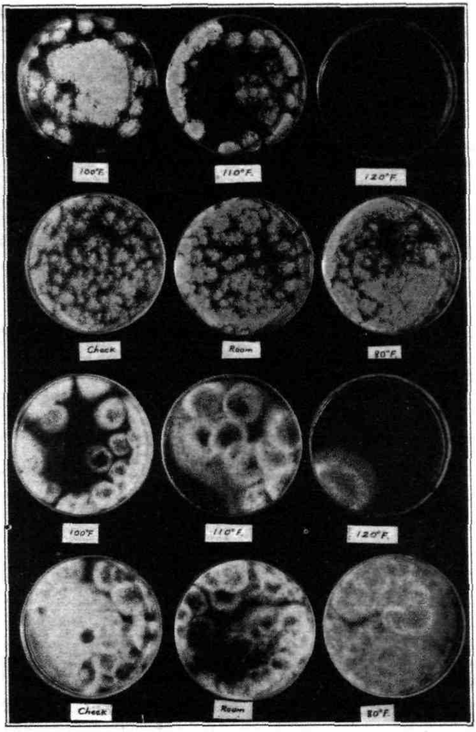


Fig. 2.—Effect of 6 per cent borax solution at various temperatures (from room temperature to 120° F), on spores of *Penicillium italicum* (upper 6 plates), and on *P. digitatum* (lower 6 plates); exposure time, 5 minutes. The plates represent a 1:100,000 dilution of the spore suspensions. The untreated spore suspensions of the two species at temperatures below 100° F yielded numerous colonies, while treatments at 120° F killed all the spores of *P. italicum* and nearly all those of *P. digitatum*.

at the same temperature for 10 minutes it is 1.2. Similar relations were found with *P. digitatum*; that is, 0.9 per cent of the conidia germinated after 110° F for 16 minutes. 3.9 per cent germinated after 110° F for 10 minutes. The results indicate that the longer the time of exposure to 6 per cent borax at the same temperature (110° F), the more effective

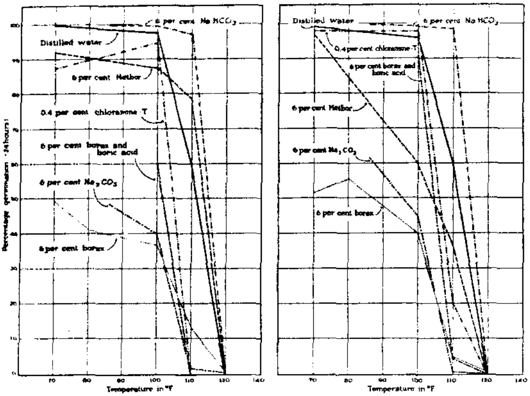


Fig. 3.—Left: Comparative effect of six solutions and distilled water at various temperatures, on the spore germination of *Penicillium italicum*: exposure time, 5 minutes. Six per cent borax-boric acid and 0.4 per cent chloramine-T at 110° F killed all the spores while 6 per cent sodium carbonate killed nearly all.

Right: Effect of the same solutions on the spore germination of *P. digitatum*. Six per cent solutions of sodium carbonate, borax-boric acid, and borax at 110° F killed nearly all the spores.

For convenience in plotting, the graphs are shown as starting at 70° F instead of 69° as shown in the tables; that temperature is still within the room-temperature range of 66°–72°, although not the exact average.

is the material in reducing both spore germination and the growth in plates of the two fungi.

Temperature Relations.—In order to determine whether the toxicity of borax is affected by higher temperatures, spores of both fungi were treated with 6 per cent borax solution for 5 minutes at the following temperatures: 66° to 72° (room temperature), 80°, 100°, 110°, and 120° F. The results are summarized in table 2, entries 53 to 64, and illustrated in figure 2.

The results show that, when the temperature increased from room tem-

perature to 120° F (49° C), the spore germination (24 hours later) decreased from 49.0 per cent to 0.1 per cent in the case of *Penicillium italicum*. In the case of *P. digitatum*, the same tendency is found, that is, germination decreased from 51.5 per cent to 1.0 per cent.

However, even in distilled water the higher temperatures are effective in decreasing germination (table 2, entries 27 to 34), though 6 per cent borax at these temperatures is seen to be even more effective, indicating

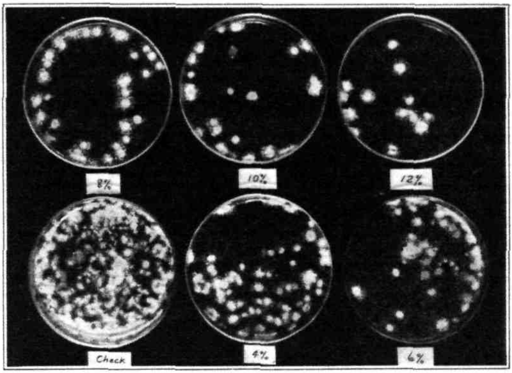


Fig. 4.—Effect of various concentrations of borax solution, at 110° F for 5 minutes, on spores of *Penicillium italicum*. The plates represent a 1:100,000 dilution of spore suspensions. The untreated suspension yielded numerous colonies, but treatments at 10 or 12 per cent killed large numbers of spores.

an intrinsic fungicidal action of the borax. Furthermore, in the case of *Penicillium digitatum* spores treated at 80° F, it seems that both germination and growth in plates are greater than at room temperature. This was true in Winston's (*41*) recent experiments. His conclusion is that increasing the temperature of borax was not ordinarily accompanied by a corresponding reduction in decay, except at 110° F. The comparative results are shown in figure 3.

Concentration.—Tests were made to determine the best concentration of borax solution. The concentration of the chemical ranged from 4 to 12 per cent in steps of 2 per cent, and the treatments were made at 110° F with a 5-minute period of exposure. The results are given in table 2, entries 65 to 76, and illustrated in figure 4.

The results show that as the concentration of borax solution increases the percentage viability of the treated spores decreases. Working on the control of decay in citrus fruit Barger (*4*) obtained a similar result. Winston (*41*) found that increasing the concentration of the borax solution up to 10 per cent, progressively decreased decay in the treated oranges; above 10 per cent, however, there was no marked increase in effectiveness of decay control.

INFLUENCE OF TEMPERATURES ON EFFECTIVENESS OF A MIXTURE OF BORAX AND BORIC ACID

From the result of experiments, Powell (*28*) suggested that the use of 2.5 to 5.0 per cent hot solutions of borax or boric acid, or a mixture of both gave complete control of green mold. This has been confirmed by Winston (*41*). He claimed, however, that boric acid, alone or in combination with borax, injured the rinds of oranges, grapefruit, and tangerines, although the effectiveness in decay control equaled that of borax.

Since borax and boric acid (2:1) solution is still commonly used in some packing-houses of California as an aid in washing as well as for better decay control, 8 tests including 2 checks were conducted at one time. For these treatments, the 6 per cent combined solution was adjusted to 100°, 110°, and 120° F and used for 5 minutes. The results are summarized in table 2, entries 77 to 84.

These results show that there is no appreciable reduction of germination at 100° F. A comparison with table 2, entries 27 to 34, shows, however, that the chemical solution is more effective than water at all three temperatures. As indicated by germination after 48 hours, the solution is more toxic to *Penicillium digitatum* than to *P. italicum*. At 110° F the combined chemical solution of borax-boric acid is slightly more toxic than is a 6 per cent borax solution; at 120° F the former is more toxic to *P. digitatum* but slightly less toxic to *P. italicum* than is the latter.

EFFECT OF METBOR

Metbor is a new material which has been mentioned by Stewart (*36*) as fully equal to borax in fungicidal efficiency, and as having very marked advantages over borax in regard to solubility in cold water and other properties. It is completely and quickly soluble in cold water in concentrations even greater than are necessary to obtain the equivalent of 8 per cent borax solution. In order to determine its effectiveness on spores of *Penicillium italicum* and *P. digitatum* as compared with borax, three separate experiments were conducted.

Time of Exposure.—This experiment consisted of 8 tests in which was used a 6 per cent solution of the chemical at room temperature (66° to 72° F) for 2, 5, 10, and 15 minutes respectively. The results are given in table 2, entries 85 to 94.

It is seen from the results that at the longest exposure time the germination after 24 hours is 65.8 per cent for *Penicillium italicum* and 49.6 per cent for *P. digitatum*. This shows much less reduction in germination than does borax under similar exposures.

Since the longest treatment was not effective, and since such treatments are impractical in the packing-house, 6 per cent Metbor has no usefulness at room temperature.

Temperature Relations.—Tests were made to determine temperature relations. Spores of both fungi were given 5-minute exposures to 6 per cent Metbor solution at temperatures of 66° to 72° F, 100°, 110°, and 120° F. The results are given in table 2, entries 95 to 104, and shown in figure 3.

In comparison with the results of the effect of temperature of distilled water (table 2, entries 27 to 34), 6 per cent Metbor is much more effective from the standpoint of spore germination, though the reduction in plate colonies is less significant.

Concentration.—Ten tests were made in which concentrations ranging from 4 to 12 per cent were used for 5 minutes at room temperature (66° to 72° F). The results are summarized in table 2, entries 105 to 116.

Although there is some reduction of germination and number of colonies at higher concentrations at 5-minute exposure, the results indicate that the chemical is not so effective in killing spores as borax.

EFFECT OF DINITRO-O-CYCLOHEXYLPHENOL

Since dinitro-o-cyclohexylphenol, used as a 0.01 per cent emulsion, has been shown to be effective in decreasing the number of brown-rot infections on lemon, caused by *Phytophthora citrophthora*, from 51.45 to 1.45 infections per fruit,[8] tests for its effectiveness on *Penicillium italicum* and *P. digitatum* were conducted, using three concentrations. The spores of the two fungi were treated separately at room temperature for 2 minutes. Results are tabulated in table 2, entries 117 to 124.

From the results it is evident that both spore germination and the growth on plates are but slightly affected by this substance under the conditions of the experiment although some reduction in number of colonies is noted as compared to the checks.

[8] Klotz, L. J., and L. L. Huillier. Dinitro-o-cyclohexylphenol as a treatment for brown rot of citrus. Unpublished data on file at Citrus Experiment Station, Riverside, California. 1936.

EFFECT OF SODIUM HYPOCHLORITE

Sodium hypochlorite (NaOCl) has been reported (*2, 3*) to be effective in controlling blue mold on apple and pear and in sterilizing packing rooms, etc. Klotz and Huillier[7] likewise found a 0.4 per cent solution completely effective in controlling brown rot of lemon.

To determine the toxicity of the chemical to blue and green molds of citrus, 6 tests were made with 0.4, 0.6, and 1.0 per cent solutions at room temperature for 2 minutes at each time; two checks were left without chemical treatment. The solutions were prepared by using a concentrated stock solution containing 6 per cent NaOCl. To prepare a solution containing 1.0 per cent available chlorine, the amount to be prepared is multiplied by 0.2. This gave the volume of stock solution to be used. The volume was made up with distilled water. The factor for 0.6 per cent is 0.111 and that for 0.4 per cent is 0.072.

The effectiveness of sodium hypochlorite is shown in table 2, entries 125 to 132. A low concentration (0.4 per cent) of the chemical in contact with the fungi for 2 minutes was completely lethal. Lack of success in some of the earlier experiments with this material was found to be due to an insufficient mixing and wetting of the mold spores.

Practically the hypochlorite has some disadvantages. Chlorine escapes, making it necessary to test and correct the treating solution frequently to maintain an effective concentration. The rate of loss of chlorine increases as the temperature of the treating solution is raised. This material is injurious to metal, cement, and wooden tanks. In the apple industry (*3*) these objections were in large measure overcome by use of a small, separate tank where the fruit was treated cold with hypochlorite solution stabilized by certain organic chemicals, and by maintaining the chlorine concentration by frequent colorimetric measurements with orthotolidine and the addition of concentrated sodium hypochlorite.

EFFECT OF SODIUM BICARBONATE

From the results of experiments in California, Barger (*5*) suggests the use of 3 to 5 per cent cold or hot solution of sodium bicarbonate for reducing mold. Negative results were obtained by Reichert and Littauer (*33*), who state that 3 and 5 per cent solutions of sodium bicarbonate for 5 and 15 minutes gave no control of wastage. Other results, however, have shown that 3 per cent and 5 per cent solutions of the chemical at 32° C

[7] Klotz, L. J., and L. L. Huillier. Sodium hypochlorite as a treatment for brown rot of citrus. Unpublished data on file at Citrus Experiment Station, Riverside, California. 1936.

(89.5° F) for 5 minutes gave some reduction of rot. Putterill and Davies (*31*) mentioned the beneficial use of 3 per cent sodium bicarbonate solution for controlling green mold. Recently Putterill (*30*) showed that 4 per cent sodium bicarbonate at high temperature was effective in controlling mold.

To determine the effect of sodium bicarbonate on spores of *Penicillium italicum* and *P. digitatum* for various periods of exposure and at several temperatures and concentrations of the chemical ($NaHCO_3$), 3 separate experiments were conducted. The effectiveness of sodium bicarbonate was also compared with that of sodium carbonate at several temperatures.

Time of Exposure.—Six tests were made with 6 per cent sodium bicarbonate at 86° F (30° C) for 2, 5, and 10 minutes respectively. The results are given in table 2, entries 133 to 140.

As indicated, a 6 per cent solution of sodium bicarbonate at 86° F and for a period of 10 minutes is practically inocuous to the spores of either mold. The plate tests show that the chemical slightly inhibited growth. These results are in contrast with those of Marloth (*22*), who found that a 6 per cent solution for 2 or 5 minutes was decidedly toxic to the spores of *P. digitatum*.

Temperature Relations.—With the aim of comparing more critically 6 per cent sodium bicarbonate solution with the same concentration of sodium carbonate (Na_2CO_3) these two substances were tested at the same time. The results are given in table 2, entries 141 to 158.

The lack of efficacy of sodium bicarbonate may be summed up by saying that the chemical at 120° F showed no advantage over water at that temperature. In fact the fungi after the bicarbonate treatment showed slightly greater viability than after the treatment in distilled water. Sodium carbonate, on the other hand, showed complete effectiveness at 120° F; and at 110° and 100° greatly reduced germination and growth.

It was also found that the temperatures of 6 per cent sodium bicarbonate solution below 120 F do not affect the viability of spores very much. This supports Barger's (*5*) conclusion that a treating temperature of 60° is as ineffective as one of 95° for this substance.

Concentration.—The effect of various concentrations of sodium bicarbonate solution was determined; the results are given in table 2, entries 159 to 166. The reduction in germination and growth of the two fungi was slight. With *Penicillium digitatum,* however, there is some reduction at 10 per cent but these tests show far less effect than is shown by the experiments of Marloth (*22*).

The results obtained by Barger (*4*) with experimentally injured and inoculated fruit show that 3 per cent sodium bicarbonate at 100° F re-

duces decay to 35.3 per cent and that 5 per cent sodium bicarbonate reduces it to 32.0 per cent. He concludes that a 3 per cent solution of sodium bicarbonate appears to be as effective in reducing mold on fruit as a 5 per cent solution.

EFFECT OF SODIUM CARBONATE

Sodium carbonate (Na_2CO_3) is used for controlling molds in many lemon packing-houses and in some orange houses in California. Doidge (*12*) has suggested the use of a 5 per cent solution of sodium carbonate for the control of *Penicillium* molds. As a result of comparative treatments of spores, Marloth (*22*) has concluded that the same concentration of carbonate is considerably more toxic than a similar concentration of bicarbonate. Recently Winston (*41*) has shown that a 3.5 per cent solution of sodium carbonate ($Na_2CO_3 \cdot 10H_2O$) at 80° F gave nearly the same result as that of the check without chemical treatment.

With the hope of obtaining more information in regard to the effectiveness of sodium carbonate in relation to time of treatment, temperature, and concentration, 3 separate experiments were conducted.

Time of Exposure.—Six tests with 6 per cent solution of sodium carbonate at 86° F were made, the spores being exposed for 2, 5, and 10 minutes.

The results shown in table 2, entries 167 to 174, indicate that the reduction of spore germination and growth in plates of both fungi become more significant as the time of exposure is extended. The results are more or less in harmony with those of Marloth (*22*).

Temperature Relations.—In this experiment, to determine temperature relations, the spores were treated with 6 per cent sodium carbonate at temperatures of 86°, 100°, 110°, and 120° F for 5 minutes. The comparative results are summarized in table 2, entries 144 to 149, and 154 to 158.

According to the results, it is shown that 6 per cent sodium carbonate at 120° F gave complete killing of spores of both fungi; that is, this treatment permitted neither germination nor the growth of a single colony.

In comparison with the results of the effect of temperature in distilled water, 6 per cent sodium carbonate at 120° F is much more effective in preventing germination than distilled water at the same temperature. This also shows that sodium carbonate apparently has an intrinsic fungicidal action on the two fungi not accounted for by the temperature of the treating solution.

It was also found that 6 per cent sodium carbonate solution at 110° F

was very toxic to both kinds of spores but this toxicity was slightly less than that at 120°.

Concentration.—In order to determine the best concentration of sodium carbonate for controlling blue and green molds, spores were treated with concentrations of 2, 6, and 10 per cent for 5 minutes at 86° F. The results are recorded in table 2, entries 175 to 182. They show that after 5-minute exposures the percentage of germination and the growth in plates of both fungi reduce significantly as the concentration increases. Under the conditions of this experiment, a 10 per cent solution of sodium carbonate was the most efficient concentration.

The results agreed with Marloth's (*22*) more in the case of *Penicillium italicum* than in that of *P. digitatum*.

A comparison of the results with those of sodium bicarbonate indicates that the same concentration of sodium carbonate was much more toxic than in the case of sodium bicarbonate.

RELATION OF TEMPERATURES TO EFFECTIVENESS OF CHLORAMINE-T

Klotz and Huillier[8] have shown that 0.4 per cent solution of chloramine-T reduces brown rot of inoculated lemons from 28.65 to 0.05 infections per lemon. To test the effect of this chemical in controlling blue and green molds, 0.4 per cent solution was used at room temperature, and at 100°, 110°, and 120° F, for 5 minutes. The effects of the various temperatures on the toxicity are given in table 2, entries 183 to 192. The reduction of spore germination and growth in plates of both fungi is very marked at higher temperatures, especially at 120° F. No germination and growth of either fungus were found at 120°. Chloramine-T is apparently more toxic to *Penicillium italicum* than to *P. digitatum* at the three higher temperatures used. At temperatures used below 110°, it showed but slight toxicity.

EFFECT OF OTHER SUBSTANCES

Sodium o-phenylphenate at 0.15 per cent has been used by Klotz and Huillier[9] in unreported experiments on the control of brown rot of lemons. They show that a 0.15 per cent solution reduces the rot of inoculated fruit from 28.65 to 0.55 infections per lemon. A commercial

[8] Klotz, L. J., and L. L. Huillier. Chloramine-T as a treatment for brown rot of citrus. Unpublished data on file at the Citrus Experiment Station, Riverside, California. 1936.

[9] Klotz, L. J., and L. L. Huillier. Sodium o-phenylphenate as a treatment for brown rot of citrus. Unpublished data on file at the Citrus Experiment Station, Riverside, California. 1936.

washing powder containing mostly soda ash with some caustic and a trace of pine oil, has also been commonly used in some of the packinghouses in California, and chloramine-T is reported to have been tried.

An experiment was set up to test the relative effectiveness of these three substances. The time of exposure was 5 minutes, the temperature, 66° to 72° F, and the concentrations as follows: sodium o-phenylphenate, 0.15 per cent; chloramine-T, 0.4 per cent; and washing powder, 1.0 per cent. The results are shown in table 2, entries 193 to 200.

Under the conditions used, sodium o-phenylphenate is the most effective substance of the three in reducing germination and the growth of *Penicillium digitatum;* it was also slightly more effective than chloramine-T and washing powder in reducing germination of *P. italicum*. The latter two substances are almost without effect on *P. digitatum* under the conditions mentioned above. With *P. italicum*, 0.4 per cent chloramine-T is more effective in reducing germination and the number of colonies than is 0.15 per cent sodium o-phenylphenate or the 1 per cent washing powder.

DISCUSSION

The inhibiting or the lethal effect of a given solution on the spores of a fungus is dependent upon a number of factors, including concentration of the fungicide and the spore suspension, duration of exposure, solvent for the fungicide, temperature, H- and OH-ion concentration, and the characteristically specific nature of the cations and anions. In the tests reported in this paper at the lower temperatures of 69°, 86°, and 100° F, and with a 5-minute treatment (2 minutes for sodium hypochlorite) the solutions in order of toxicity from highest to lowest are as shown in table 3, the numbers in the fourth column being the relative viability of the treated spores based on the combined mean of the percentage of germination after 24 hours and the viability on a culture medium.

The sodium hypochlorite solutions acting for only 2 minutes were fatal to the spores of both *Penicillium italicum* and *P. digitatum*. These results are similar to those of Baker and Heald (*3*) who found that rinsing apples for one minute with a sodium hypochlorite solution containing 0.4 per cent available chlorine was very effective to reducing the number of viable spores of *P. expansum* on the surface and in the lenticels, and in decreasing losses from decay by this organism.

Sodium carbonate occupies a relatively high position in the toxicity tables, and shows a greater effect on *Penicillium italicum* than on *P. digitatum*.

Borax in the cool solutions was more toxic to *P. digitatum* than to *P.*

TABLE 3

AVERAGE VIABILITY OF THE SPORES AFTER TREATMENT WITH VARIOUS FUNGICIDES AT VARIOUS TEMPERATURES

Penicillium italicum				*Penicillium digitatum*			
Order of toxicity of fungicide	Fungicide	Concentration of fungicide, per cent	Viability*	Order of toxicity of fungicide	Fungicide	Concentration of fungicide, *per cent*	Viability*
			Treatment at 69°, 86°, and 100° F				
1	Sodium hypochlorite†	1.0	0.0	1	Sodium hypochlorite†	1.0	0.0
2	Sodium hypochlorite†	0.6	0.0	2	Sodium hypochlorite†	0.6	0.0
3	Sodium hypochlorite†	0.4	0.0	3	Sodium hypochlorite†	0.4	0.0
4	Sodium carbonate	6.0	30.4	4	Sodium o-phenylphenate	0.15	7.3
5	Sodium o-phenylphenate	0.15	38.2	5	Castile soap for 10 minutes followed by borax	0.25 6.0	 33.8
6	Castile soap for 10 minutes followed by borax	0.25 6.0	 58.4	6	Sodium carbonate	6.0	48.3
7	Chloramine-T	0.4	58.0	7	Borax	6.0	56.5
8	Borax-boric acid (2:1)	6.0	58.15	8	Metbor	6.0	78.1
9	Borax	6.0	58.17	9	Sodium bicarbonate	6.0	87.7
10	Dinitro-o-cyclohexylphenol†	0.001	70.1	10	Dinitro-o-cyclohexylphenol†	0.001	88.2
11	Metbor	6.0	74.7	11	Washing powder	1.0	91.2
12	Washing powder	1.0	79.7	12	Borax-boric acid (2:1)	6.0	94.1
13	Sodium bicarbonate	6.0	83.9	13	Chloramine-T	0.4	93.5
			Treatment at 110° and 120° F				
1	Chloramine-T	0.4	0.1	1	Sodium carbonate	6.0	0.6
2	Sodium carbonate	6.0	0.5	2	Chloramine-T	0.4	10.4
3	Borax-boric acid	6.0	4.6	3	Borax-boric acid	6.0	11.1
4	Borax	6.0	19.3	4	Metbor	6.0	20.6
5	Metbor	6.0	31.1	5	Borax	6.0	22.4
6	Sodium bicarbonate	6.0	34.8	6	Sodium bicarbonate	6.0	44.1

* Calculations all based on percentage germination after 24 hours and relative viability, being related to germination and relative viability of water check (100).

† All treatments for 5 minutes except in the case of dinitro-o-cyclohexylphenol and sodium hypochlorite treatments of 2 minutes.

italicum, although the borax-boric acid mixture had a greater effect on the latter.

Sodium o-phenylphenate was effective on both fungi but more toxic to *Penicillium digitatum*.

Cold 6 per cent solutions of Metbor and of sodium bicarbonate were relatively ineffective on the two fungi. A 0.4 per cent solution of chloramine-T decreased the viability of *P. italicum* 42 per cent but *P. digitatum* only 7 per cent.

At the higher temperatures of 110° and 120° F, and with a 5-minute treatment (6 minutes at 110° F in one experiment with borax), the order of toxicity of the several solutions is shown in table 3.

One of the striking features of this record is the toxicity positions occupied by the warm chloramine-T and borax-boric acid solutions as compared with those of the solutions at the lower temperatures. Another surprising result is the consistently greater resistance of *Penicillium digitatum* to the growth inhibition of the several solutions. This would indicate that *P. digitatum* would be found more difficult to control with the solutions mentioned above than would *P. italicum*. But most of the results of experiments on decay prevention in citrus fruits show that borax or borax-boric acid is much more effective against the green than against the blue mold. As one possible explanation for this difference may be offered the suggestion that when the solution comes in contact with the oil of the rind, some chemical action takes place whereby the original specific toxicity of the treating solution to the particular spores is altered. Another explanation may be that some of the treating substances remaining on the rind surface or in injuries may react differently to germinated spores of one fungus than to those of the other. Marloth (*22*) found that germinated spores of both fungi were more readily killed by borax, sodium carbonate, and sodium bicarbonate than nongerminated spores.

The comparative results are shown in figure 3. Sodium bicarbonate again shows the least toxicity, while sodium carbonate averages higher for the two fungi. The importance of temperature is readily seen from the above and from a consideration of the results in table 2. The lower toxicity at the lower temperatures may be due in part to poor wetting of the spores, although that factor was decreased as much as possible by the pretreatment with soap solution. The greater toxicity of the chemical solutions at the higher temperatures should be largely related to the increased velocity and penetrating power of the toxic ions and molecules and to the kinetic energy of the particles of the solvent itself, since water at 120° F greatly inhibited subsequent germination. A 5-minute exposure

to sodium carbonate, borax-boric acid mixture, chloramine-T, and Metbor at 120° F was fatal to the spores of the two fungi, while water, sodium bicarbonate, and borax at the same temperature and exposure permitted survival of a small percentage. At 110° F nearly all the spores of *Penicillium italicum* were killed by chloramine-T and the borax-boric acid mixture. Not all the spores of *P. digitatum* were killed by any of the solutions tried at 110° F, although 6 per cent sodium carbonate permitted only 0.4 per cent germination after 24 hours and a relative viability of only 1.9.

As would be expected, the longer the period of exposure to any of the several chemicals used, the smaller the percentage of germination of either fungus; this is especially striking in the experiments with 6 per cent borax solution at 110° F. The comparative results are given in figure 5.

The results of the concentration experiments show that all the spores of both blue and green molds were killed at room temperature by the weakest solution (0.4 per cent) of sodium hypochlorite tried. After treatment with 12 per cent borax solution at 110° F, no spore germinations were observed after 24 hours. Conidia of *Penicillium italicum* treated with 8 per cent borax solution at 110° F were affected significantly, while those of *P. digitatum* were affected significantly in 10 per cent borax solution at the same temperature. A 10 per cent solution of Metbor at 66° to 72° began to reduce the germination of *P. italicum* markedly. In the case of *P. digitatum* spores were affected significantly in 12 per cent Metbor solution at 66° to 72°. From this point of view, it is also indicated that *P. digitatum* is more resistant to the toxicity of higher concentrations of borax and Metbor than *P. italicum*. Both spores began to be affected by a 10 per cent sodium carbonate solution at 86° F. Spores of *P. digitatum* and *P. italicum* were but slightly affected by a 10 per cent solution of sodium bicarbonate at 86° F, although the former were less inhibited than the latter. The differences among concentrations of dinitro-o-cyclohexylphenol as to their effect upon spores of both fungi were not sufficiently great to be of practical significance.

As far as the results of these experiments are concerned, it may be concluded that the most effective and economical solution for killing blue and green mold spores is the 0.4 per cent sodium hypochlorite used for 2 minutes at room temperature. The following solutions may also be effective when used at 120° F for 5 minutes: 0.4 per cent chloramine-T, 6 per cent mixture of borax and boric acid, 6 per cent sodium carbonate, and 6 per cent Metbor. Eight to 12 per cent solutions of borax are effective if used at 110° for 14 to 16 minutes.

As Fawcett (*14*) has pointed out, 120° F for 2 to 4 minutes is gener-

ally considered the danger point for the temperature of washing or treating solutions. With freshly picked fruit, especially with lemons, it is

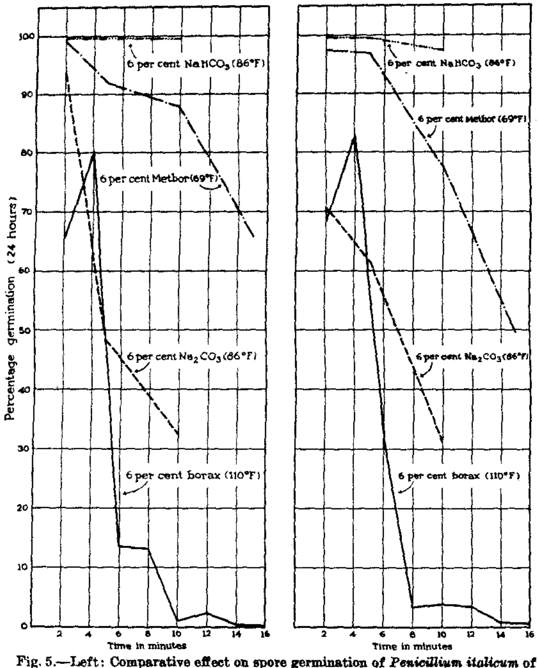


Fig. 5.—Left: Comparative effect on spore germination of *Penicillium italicum* of various exposures to four different solutions at recommended temperatures. Six per cent borax solution at 110° F for 10 minutes or longer killed nearly all the spores.
Right: Effect of the same solutions on the spore germination of *Penicillium digitatum*. Six per cent borax solution at 110° F for 8 minutes or longer killed nearly all the spores.

necessary to use lower temperatures or to dry the fruit for several days before treatment, as pointed out by Fawcett and Klotz (*16*). To avoid injury to freshly picked fruit and at the same time secure partial control

of blue and green molds the four solutions mentioned above may be used at 110° to 115° F, providing the fruit is first allowed to dry 3 to 5 days.

The spores showed a great tolerance toward hydroxyl ions. A 5-minute treatment in 2 per cent Castile soap having a pH 10.05 had no effect on the subsequent viability of the spores. A 2-minute treatment in 6 per cent soda ash solution (pH 10.16) and a 5-minute immersion in a solution of 1 per cent washing powder (pH 9.90) had but slight effect on germination. *However, a 2-minute exposure to the 0.4 per cent alkaline sodium* hypochlorite solution (pH 11.1) was fatal. While the high OH-ion concentration was likely an important factor in the toxicity of the hypochlorite solution, the lethal effects may have been due in a large measure to the toxicity of free chlorine, OCl ions, and nascent oxygen. Klotz (*21*) has shown that clorine even in small concentration is lethal to *Penicillium italicum* and *P. digitatum*. Marloth (*22*) found that the spores of the two fungi showed abundant germination in a slightly buffered Duggar's solution to which orange extract had been added and which had been adjusted to the acidity-alkalinity range of pH 2.4 to pH 9.4. In the range pH 3.0 to pH 9.2 the germination in that medium was relatively indifferent to H-ion concentration. No germination of either organism was obtained in Sörensen's glycocoll buffer above pH 9.7 or in 2.6 and 10.0 per cent solutions of sodium carbonate, potassium carbonate, sodium bicarbonate, and potassium bicarbonate, or in 4 per cent sodium tetraborate solution. Since the estimations of H-ion concentration in those studies were made with the quinhydrone electrode they are reliable only up to approximately pH 7.5. For example, the pH of 10 per cent solutions of bicarbonate and carbonate which were reported as 8.6 and 11.4, respectively, were estimated by the glass electrode and reported in the present paper as pH 8.0 and pH 9.9.

The results seem to indicate that $\overset{+}{H}$ and $\overset{-}{OH}$ ions in the concentration range of pH 2.4 to pH 10.0 are in themselves relatively innocuous to the spores of *P. italicum* and *P. digitatum*. However, owing to their possible effect in altering the permeability of the fungus protoplasts these very mobile ions may affect the results with other toxic ions. Osterhout (*25*, *26*) found that alkali increases permeability, and that acid at first decreases then rapidly increases permeability in the seaweed *Laminaria saccharinum*.

Unlike the technique of the former paper (*22*) in which the spores were germinated directly in media whose pH was adjusted, the procedure of the present paper exposed the spores for only a short period to the action of the $\overline{OH}$ and other ions, then rinsed with water and mounted them in a medium favorable for germination. The short period of exposure to

the toxic solution followed by a rinse would correspond to that treatment usually given in a packing-house. Some packing-house procedures, however, as in the case of the water-wax method for lemons, allow the toxic solution, containing soda ash in this case, to dry on the fruit and thus maintain a protective coating. As suggested by Marloth (*22*) the toxic salt thus deposited would form a relatively concentrated solution in condensation water that might subsequently form on the surface of the fruit and would, by killing the tender swollen spores and germ tubes, repulse invasion of the fungi that might lodge in that water.

Some discrepancies are seen between the results of the germination tests and those of the dilution plate methods. These may be due to the clumps of spores forming individual colonies which would be recorded as arising from single spores.

SUMMARY

To obtain information on the toxicity of various chemical solutions, at several temperatures and concentrations, to *Penicillium italicum* and *P. digitatum* (the causal agents of blue and green mold of citrus fruits), the spores of the fungi were immersed for certain time periods, and their subsequent viability compared with that of untreated spores by means of germination and dilution-plate tests. The technique of the methods employed is described in detail.

It was shown that a 0.25 per cent solution of a nontoxic soap effectively wets and prepares the spores of *Penicillium italicum* and *P. digitatum* for the chemical treatment that follows. No decrease in germination followed the pretreatment with the soap.

Distilled water at 120° F for 5 minutes killed approximately 90 per cent of the spores.

Tests in which 6 per cent borax at 110° F was used for 2, 4, 6, 8, 10, 12, 14, and 16 minutes, and at room temperature (66° to 72°), 80°, 100°, 110°, and 120° F for 5 minutes, and at concentrations of 4, 6, 8, 10, and 12 per cent for 5 minutes at 110° F, showed, as would be expected, that the longer the exposure to, the higher the temperature of, and the greater the concentration of the chemical, the more effective was the solution in reducing viability. Similar relations were found with sodium carbonate and Metbor.

Under the conditions of the experiments toxicity of the several solutions to spores of *Penicillium italicum* and *P. digitatum* was more dependent on temperature than on concentration of the chemicals or the period of immersion. A 5-minute exposure at a temperature of 120° F

in a 6 per cent borax-boric acid mixture, or 6 per cent Metbor, or 0.4 per cent chloramine-T, or in 6 per cent sodium carbonate, was lethal to the spores of both fungi. Details of the effects of the several temperatures may be secured from tables 2 and 3.

A saturated solution of dinitro-o-cyclohexylphenol and a 1 per cent proprietary washing powder used at room temperature for 2 minutes and 5 minutes, respectively, showed only a slight inhibitory effect on spore germination.

A 5-minute exposure of the spores in 6 per cent sodium bicarbonate at 86°, 100°, 110°, and 120° F showed no advantage of the chemical over water. At 86° F, immersion in a 10 per cent solution of sodium bicarbonate for 5 minutes or in one of 6 per cent for 10 minutes, had but little effect on the spores.

Two-minute exposures to 0.4, 0.6, and 1.0 per cent solutions of sodium hypochlorite were fatal to the spores of both fungi.

Excluding the sodium hypochlorite solutions which killed all the spores of both fungi, the three most efficacious solutions, when used at 100° F and below for 5 minutes, were 6 per cent sodium carbonate, 0.15 per cent sodium o-phenylphenate and 6 per cent borax; at 110° and 120° F the 3 most toxic were 0.4 per cent chloramine-T, 6.0 per cent sodium carbonate, and the 6 per cent mixture (2:1) of borax-boric acid.

ACKNOWLEDGMENTS

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LEAF-SCAR INFECTION IN RELATION TO THE OLIVE-KNOT DISEASE

WM. B. HEWITT

LEAF-SCAR INFECTION IN RELATION TO THE OLIVE-KNOT DISEASE[1,2]

WM. B. HEWITT[3]

INTRODUCTION

OLIVE KNOT, a serious disease of *Oleo europea* L. in most of the olive-producing districts north of the Tehachapi mountains of California, is characterized by the development of overgrowths, knots on the branches. These knots develop most frequently at the leaf scars except in years when freezing injury occurs. The present study furnishes inoculation data, with histological evidence that the scars, under certain conditions, are portals of entry of the causal agent, *Bacterium savastanoi* E.F.S., into the host. This paper also deals with inoculation experiments used to determine the length of time during which the leaf scars are susceptible to infection. It further describes the microchemical and histological studies of the development of the abscission region just before and after leaf fall, and thereby elucidates the rôle played by leaf scars in infection.

PREVALENCE OF LEAF-SCAR INFECTION

Observations in California olive groves show that a large percentage of the new knots forming each year develop at leaf scars. Horne, Parker, and Daines (*2*)[4] were first to point out this fact: "By far the largest number of knots appear on leaf scars or wound callus." Wilson (*6*) also recognizes the importance of leaf scars as infection courts. He points out that in years other than those in which freezing injury occurs, as high as 90 per cent of the new knots on branches develop at leaf scars.

The distribution of inoculum from active knots necessary to infect leaf scars depends upon the presence of dripping moisture, as from rain. Horne, Parker, and Daines (*2*) state that the bacteria exude in a slimy mass from the fissures of knots during rain. Wilson (*6*), in further and more extensive studies, demonstrates the importance of rain in the exudation and spread of the organism as related to infection. He shows that enough bacteria were present to cause infection within a short period after the knots were moistened. He placed wounded, healthy, potted trees under diseased trees, allowed a fine spray of water to fall over them,

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[2] A thesis submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy, University of California, 1936.

[3] Junior Plant Pathologist in the Experiment Station.

[4] Italic numbers in parentheses refer to "Literature Cited" at the end of this paper.

and then placed the potted trees in the greenhouse so that the knots might develop. In this experiment he reports numerous knots developing on trees removed from the spray 7 minutes after it was started. Wilson also infected newly formed leaf scars by artificial inoculation and showed

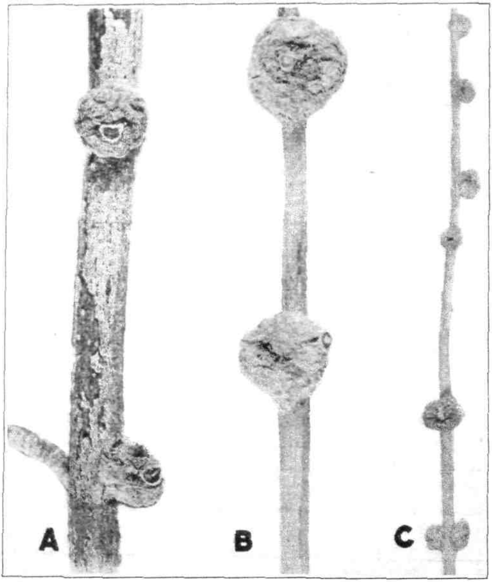

Fig. 1.—*A*, *B*, Olive knots showing leaf scars on the surface. *C*, Young olive knots resulting from natural leaf-scar infections. (*A*, ×2; *B*, ×4; *C*, natural size.)

that the vessels in the leaf scars were open at the time of leaf fall. According to his climatic studies, infection may occur in a range of temperature from about 40° to 100° F.

The origin of the knots can be determined by examination. Those developing at the nodes usually form at the leaf scar, which upon close inspection, can usually be seen on the surface of young knots (fig. 1).

To gain further evidence as to the importance of leaf-scar infection, the following experiments were carried out. On May 5, 1936, in a Mission-olive orchard in Sacramento County, 60 healthy branches were tagged. When these were re-examined in October, 241 knots were discovered, of which 225 (about 93.5 per cent) were located at leaf scars.

On May 14, 1936, in the same olive orchard 339 yellow leaves about ready to fall were removed by bending them slightly backward. This leaf removal was continued until interrupted by a shower. Other light showers occurred on May 28. By August, 229 of the leaf scars (67.5 per cent) had developed knots.

The olive normally drops some of its older leaves each season, and the time of abscission of leaves plays an important part in the infection of leaf scars. Wilson (*6*), studying natural leaf fall, finds that the period of maximum fall varies with the season; and, in unpublished experiments, shows that leaf drop generally starts about the time growth begins in late January or February, rises slowly during the early months, and increases suddenly to a maximum at full bloom around the middle of May. It then decreases through the summer and usually ceases by the latter part of September. Observations by the writer during the seasons of 1935 and 1936 confirmed those of Wilson.

MATERIALS AND METHODS

Choosing Material.—All experiments, unless otherwise stated, were conducted on the Mission variety of olive in an orchard in Sacramento County, California.

Fresh leaf scars were made by removing only the leaves that were three-fourths to entirely yellow. In such leaves the abscission process was well advanced, and the leaves were about to drop. The leaves were removed by placing a pencil or finger at the tip of the leaf and bending them toward the base of the stem. If the leaf fell by the time it was pushed back one half of the distance to the main limb—that is, approximately 45°—the resulting scar was encircled with a red wax pencil mark and retained for use in these studies.

Method of Inoculation.—In artificial inoculations a water suspension of *Bacterium savastanoi* E.F.S. from a 36- to 48-hour culture on potato dextrose agar was used. A drop of this suspension was placed over the leaf scar with a small camel's hair brush.

Collecting Leaf Scars.—Leaf scars were collected by cutting out a small portion of the stem along with the leaf scar (fig. 2, *B*).

Moist Chambers.—Moist conditions were provided by enclosing a

branch in a cylinder of wire gauze (⅛-inch mesh screen) and afterwards wrapping it with three layers of wet cheesecloth. The bottom of the chamber was then placed in a can containing water, and the can was suspended by a wire from a large branch in the tree (fig. 2, *A*).

Method of Holding Fresh or Killed Unimbedded Leaf Scars for Sectioning.—Sliding microtome sections of leaf scars killed, fixed, and stored in formalin-acetic-alcohol solution, together with sections of fresh

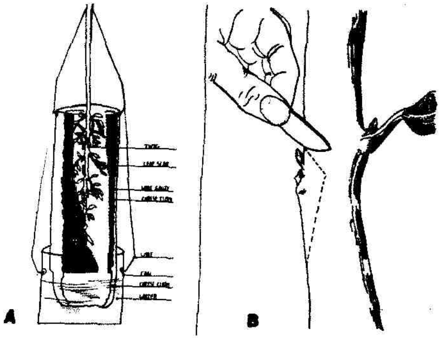

Fig. 2.—*A*, Diagram of the moist chamber used. *B*, Illustrating the method used for cutting leaf scars from the stems before fixation.

leaf scars, were used for these studies. The collected scars were small and in order that they might be sectioned without imbedding, a method was devised to hold the scars. They were mounted directly in paraffin blocks. The tissue was first blotted until the surface appeared dry. A small hole was melted in one end of a paraffin block with a hot iron rod, and the scar was then inserted into the melted paraffin. To loosen air bubbles adhering to the scars, a warm dissecting needle was moved around the material; this also served to orient the scars in the blocks. The paraffin block was then cooled in ice water, trimmed, and sectioned by the sliding microtome. Most of the sections of the leaf scars freed themselves from the surrounding paraffin when placed in water, but sections that adhered to the paraffin were freed easily by prodding with a small brush.

Paraffin Method Used.—The material was killed and fixed in a solution of formalin 10 cc, acetic acid 10 cc, and 50 per cent alcohol 100 cc for 12 hours or longer. It was imbedded in paraffin as follows: (A) The speci-

mens were transferred directly from the formalin-acetic-alcohol into a solution of 10 per cent glycerin in water. The solution was then allowed to evaporate until it became thick. (B) The material was next successively transferred through the following glycerin and normal butyl alcohol mixtures and left in each for 48 to 72 hours:

(a) 75 parts of glycerin and 25 parts of normal butyl alcohol.
(b) 50 parts of glycerin and 50 parts of normal butyl alcohol.
(c) 25 parts of glycerin and 75 parts of normal butyl alcohol.
(d) Pure dehydrated normal butyl alcohol; four changes—the first two 24 hours apart, the next two 48 hours apart—were used. The containers were placed on top of the paraffin oven to warm; this hastened the removal of all the glycerin.

(C) Small amounts of paraffin were added to the last change of butyl alcohol while the specimens were still on top of the oven, and they were allowed to stand for 12 hours. Then the process of infiltration was completed as in standard schedule (*5*).

INOCULATION OF THE ABSCISSION ZONE BEFORE LEAF FALL

If infection of the leaf abscission zone were to precede leaf fall, it might be expected to occur through a break in the epidermis or some other open infection court. This point was tested by inoculating the base of the petiole with a water suspension of *Bacterium savastanoi*. On May 31, 1935, 50 yellow leaves were inoculated at the base of the petiole. When examined on August 31, only 2 out of the 50 leaf scars had developed knots. Again on May 4, 1936, the base of the petioles of 67 yellow leaves were inoculated. The number of leaves that had fallen at intervals after inoculation were as follows: at the end of 1 hour, 29; 3 hours, 42; 24 hours, 55; and 48 hours, 64. By September 1, 2 of the 67 leaf scars had developed knots. The leaves from the leaf scars that developed knots probably fell shortly after inoculation, which allowed the inoculum to spread over the scar and accomplish infection.

The condition of the epidermis of the petiole in the region of the abscission zone was studied in 25 yellow leaves. A break that might serve as an infection court was found at the abscission zone in the axil of only one leaf. Figure 3 shows a photograph of this break. Judging from these results and from the inoculations previously described, infection of leaf scars is not apt to occur before leaf fall.

THE NEWLY FORMED LEAF SCAR

A microchemical and histological study of the abscission zone of 25 yellow Mission-olive leaves was made before and after leaf fall to determine what changes take place in this zone, because the structure and composition of these tissues were suspected to have an important relation to the infection process.

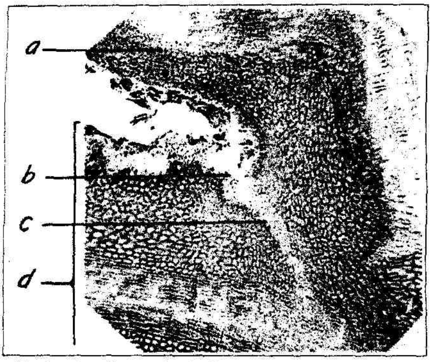

Fig. 3.—A portion of a longitudinal section through the abscission zone of a yellow leaf, showing a break in the epidermis in the axil of the leaf. Details are: *a*, Bud; *b*, a break in the epidermis at the edge of the abscission zone in the axil of the leaf; *c*, abscission zone; *d*, leaf petiole. (×55.)

The mode of abscission in the olive leaf resembled that of *Castanea sativa* Mill., as described by Lee (*4*).

The abscission zone of the olive leaf lies at the base of the leaf petiole very close to the stem and, in longitudinal sections, stains lighter than the surrounding tissues (fig. 3). The number of layers of cells involved in the abscission zone varies from 1 to 7, but is more frequently 3 to 5. The zone extends from the epidermal cells through the entire diameter of the petiole and involves all the living cells. Leaf fall does not occur until the leaf is almost completely yellow. Separation takes place along an irregular plane between the walls of cells in the portion of the zone proximal to the stem.

In no leaves studied were there any cell divisions before separation,

nor any protective layers of lignin, gum, or suberin formed in the tissues above or below the abscission zone. The cuticle, sieve tubes, vessels, and fibers were mechanically broken at leaf fall. The epidermal cells at the separation plane of the scar appeared as though they had been broken at leaf fall. Just after leaf fall, therefore, the scar was an open wound, with exposed unprotected tissue, and open vessels.

SUSCEPTIBILITY OF NEWLY FORMED LEAF SCARS TO INFECTION

In June, 1935, leaf scars made by removing yellow leaves were inoculated immediately after their formation. Some were placed in the moist chambers for incubation; others were left exposed to outside atmospheric conditions. The inoculations were made when the outside air temperature was 28.5° C in the shade and when a gentle north wind was blowing. The temperature in the moist chambers ranged from 20° to 21° C. Of the 196 leaf scars inoculated and placed in moist chambers for 24 hours' incubation, 156 (or 79.6 per cent) developed knots; and of the 224 left outside of moist chambers 184 (or 82.6 per cent) developed knots. The data show that inoculations made immediately after leaf fall produced a high incidence of leaf-scar infection, and indicate that moist chamber conditions are not essential to a high degree of infection.

PERIOD DURING WHICH LEAF SCARS ARE SUSCEPTIBLE TO INFECTION

The period during which leaf scars are susceptible to infection was determined by periodic inoculations after scars were formed.

The leaf scars of one series were left outside of moist chambers until inoculation at which time they were placed in moist chambers for incubation and left 24 hours, after which the moist chambers were removed. The leaves for a second series were removed on the same dates, the scars being placed in moist chambers when formed, to determine the influence of high atmospheric humidity on scar healing and its relation to infection. After the moist chambers had been removed a short time for inoculation, they were replaced and left over the scars for 24 hours after inoculation, then removed.

Groups of leaf scars in each series were inoculated at intervals after they were formed. Series of inoculations were made during June, 1935; made again in May, 1936, because of an early season; and repeated at shorter intervals in June, 1937. The scars were allowed to remain on the

trees until knots were well developed (fig. 1). The results are given in table 1, and presented graphically in figure 4.

As shown in figure 4, the percentage of leaf scars susceptible to infection decreased rapidly within the first day. This drop is much more pro-

TABLE 1

RESULTS OF INOCULATIONS OF LEAF SCARS AT VARIOUS TIME INTERVALS AFTER LEAF REMOVAL

Age of leaf scar at time of inoculation (*days*)	*Inoculations of 1935*		*Inoculations of 1936*		*Inoculations of 1937*		
	Number of leaf scars inoculated	Per cent of leaf scars forming knots	Number of leaf scars inoculated	Per cent of leaf scars forming knots	Age of leaf scar at time of inoculation (hours)	Number of leaf scars inoculated	Per cent of leaf scars forming knots
Scars left outside moist chambers, except for 24-hour inoculation period							
0	11	81.8	46	97.9	0	61	96.5
1	14	57.2	37	64.8	1	36	100.0
2	12	41.6	45	57.8	3	24	99.2
3	12	25.6	38	39.5	6	24	79.2
4	10	40.0	33	42.4	12	22	68.2
5	12	33.0	44	34.1	24	23	52.2
7	12	8.3	37	13.5	48	23	42.4
8	..	....	40	15.0	72	35	21.7
9	..	....	14	7.0	96	45	28.9
10	..	....	12	0	..	..	....
13	11	0	..	....	..	..	....
15	12	0	..	....	..	..	....
21	10	0	..	....	..	..	....
Uninoculated	15	0	34	14.7	Uninoculated	25	0
Scars kept in moist chambers until 24 hours after inoculation							
0	20	85.0	80	90.0	0	61	96.5
1	24	29.2	74	24.3	1	25	92.0
2	30	3.3	64	17.2	3	20	100.0
3	39	5.1	39	12.9	6	20	85.0
4	16	6.2	98	9.2	12	20	75.0
5	10	0	60	1.7	24	22	18.2
6	..	....	25	0	48	22	13.6
7	16	0	..	....	72	24	8.0
11	11	0	..	....	..	..	....
Uninoculated	10	0	51	....	Uninoculated	15	0

nounced in the scars kept in moist chambers than in the scars left outside. According to these data, leaf scars left outside of moist chambers are susceptible to infection longer than those kept in moist chambers. The latter became immune about the fifth day, whereas the former did not

become immune until they were 7 to 9 days old. The infection of some of the scars used as controls invalidates the curve of 1936 inoculations after the sixth day, since any infection shown after this time may have resulted from earlier natural infection that prevented the curve from dropping to zero. The United States Weather Bureau in Sacramento recorded a

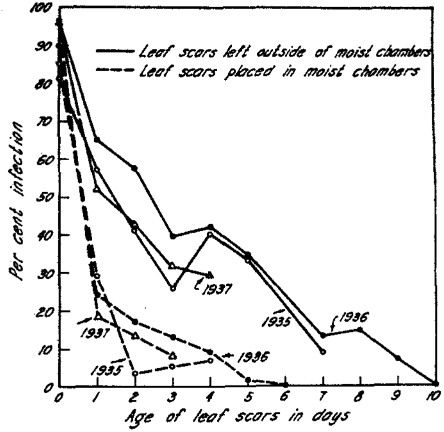

Fig. 4.—Showing the relation between the age of the leaf scars in days at the time of inoculation with *B. savastanoi* and the per cent of scars developing knots for both those kept in moist chambers and those left outside.

trace of rain on May 13 and 0.30 inch on May 14. These rains, occurring on the fifth and sixth days of the series of inoculations, might have initiated some infection in the experimental scars.

In 1937 the inoculations were made at shorter intervals to discover when the drop in infected scars takes place through the first 24 hours (table 1 and fig. 5). During the first 12 hours, the percentage of scars infected at each inoculation period remained about the same, both for those scars kept in moist chambers and for those left outside. In the former, the infected scars dropped from 75 per cent at the 12-hour period to only 18 per cent at the 24-hour period, while in the latter group the percentage of scars infected dropped gradually at each inoculation after 3 hours.

Though the number of scars used in any one year's inoculation is small, the results for the three years consistently show that leaf scars soon become immune to infection (fig. 4).

If the conditions within the moist chambers might simulate a rather long period of high humidity, such as might occur with a rain of a week's

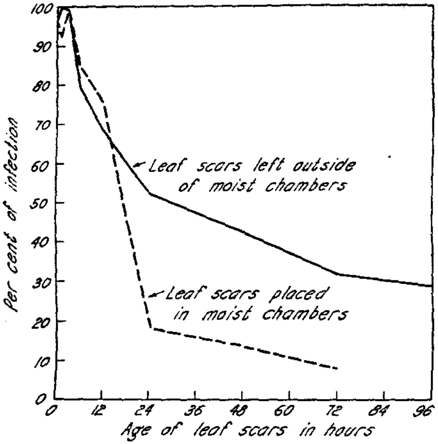

Fig. 5.—Results of the 1937 inoculations of leaf scars at short time intervals after leaf removal, showing the relation between the age of the leaf scars in hours at the time of inoculation and the per cent of scars which developed knots, for both those placed in moist chambers and those left outside.

duration, then, in the early summer the processes that prevent infection take place more rapidly during periods of continuous high humidity than during those of low humidity. Under California conditions, however, the spring rains are usually short, generally lasting only a few hours but seldom continuing more than two days. The leaf scars left outside the moist chambers were subjected, then, to the weather conditions that most frequently occur in the spring. Apparently, therefore, 80 to 95 per cent of the leaf scars are susceptible to infection at the time of leaf fall, the percentage of infectable scars drops to about 40 per cent by the fourth day, and most scars are immune to infection by the end of the ninth day after leaf fall.

MICROCHEMICAL STUDIES OF THE HEALING PROCESSES IN LEAF SCARS

Microchemical studies of the healing processes of uninoculated leaf scars were made to determine the processes which take place in these tissues and which may explain the results obtained in the inoculation experiments. For this purpose leaf scars were formed by removing yellow leaves, but the scars were not inoculated. One group was placed in moist chambers; the other left outside of moist chambers. A collection from each group was made when the leaf scars were formed, and other collections of ten scars each were made on each of the ten days following. The scars were preserved in the alcohol-formalin-acetic acid fixing solution until they were sectioned. The healing processes of the scars used in these studies should correspond to those of the leaf scars used in the inoculation work, for these collections came at intervals corresponding to the inoculation intervals which were made in experiments to determine how long the scars were susceptible to infection.

Longitudinal sections about 15 microns thick were made with a sliding microtome. Microchemical tests consisted of determining the presence of and changes in wound gum, water-soluble gums, lignin, suberin, oil, starch, and tannins. Unless otherwise stated, the microchemical methods used were those described by Rawlins (*5*).

Wound Gum.—Wound gum is defined as a substance often found in vessels of plants adjacent to wounds and in wood invaded by wood-decay fungi. It is insoluble in water and stains red with phloroglucinol in hydrochloric acid (*5*). Haas and Hill (*1*), in a similar description, mention the following properties of wound gum: It does not swell in water; it is insoluble in sulfuric acid and caustic soda; and on oxidation it yields both mucic and oxalic acids. According to Küster (*3*) wound gum is insoluble in alcohol, ether, carbondisulfide, cold nitric acid, and cold aqua regia but soluble in warm nitric acid and in a combination of hydrochloric acid and chlorate of potash.

Table 2 gives the results of microchemical tests in leaf-scar tissues. The formation of wound gum was one of the first and most conspicuous processes observed in the healing of leaf scars. It occurred in the cell walls, intercellular material, intercellular spaces, and lumina of vessels.

The only test that would distinguish wound gum from lignin was the Maule reaction (*5*), which colors lignified tissues light red but does not give a color reaction with wound gum in the leaf scar. Wound gum, therefore, is regarded in this paper as the water-insoluble material that forms

in the leaf-scar tissues, reacts positively to most lignin tests, but does not color with the Maule reaction.

Longitudinal sections of leaf scars killed and fixed immediately after they were formed exhibited no detectable change in the composition of the tissues except in one scar where a trace of wound gum was found in

TABLE 2

MICROCHEMICAL REACTIONS OF THE WOUND-GUM ZONE IN THE LEAF SCAR

Classifications concluded on Opposite Page

Chemical test	Tissues adjacent to the wound-gum zone				
	Reaction of the cell walls of:				Inter-cellular material of parenchyma
	Cortex parenchyma	Cortex fiber cells	Xylem parenchyma	Vessels	
Iodine and potassium iodide	—*	Yellow	—	Yellow	—
I-KI followed by 72 per cent H_2SO_4	Blue	Yellow, later brown	Blue	Yellow, later brown	—
Zinc chloriodide	Blue	Light clear yellow	Blue	Light clear yellow	—
Ferric chloride and potassium ferricyanide	Green	Light blue	Green	Light blue	Dark green
Dinitro-phenylhydrazine	—	Yellow	—	Yellow	—
Maule reaction	—	Light red	—	Light red	—
Phloroglucinol in HCl	—	Red	—	Red	—
Orcinol followed by HCl	—	Light blue	—	Light blue	—
Ruthenium red‡	—	—	—	—	—
Phloroglucinol in HCl‡	—	—	—	—	—
Zinc chloriodide‡	Blue	Blue	Blue	Blue	—
Ruthenium red¶	Light red	Red	Red	Light red	Red
Phloroglucinol in HCl¶	—	—	—	—	—
Zinc chloriodide¶	Blue	Blue	Blue	Blue	—
Polarized light	Anisotropic	Anisotropic	Anisotropic	Anisotropic	Isotropic

* The dashes indicate negative results.
† The substances were removed by chlorination.
‡ After chlorination and sodium-sulfite treatment.
¶ After chlorination and treatment with 10 per cent ammonium hydroxide.

cortical cells. The first change regularly noted after the scars were formed was in the protoplasm of the cells that were to make up the wound-gum zone. Such protoplasm contained a yellow amorphous material (table 4) which stained a dark brown when treated with phloroglucinol in hydrochloric acid.

Traces of wound gum were found at the end of the first day in the tissues of scars left outside moist chambers (table 3 and plate 1, *A*), but not until the second day in the tissues of the scars placed in moist chambers (table 5 and plate 3, *A*). In the former the wound-gum zone developed in 3 to 6 layers of cells, which were usually from 4 to 11 tiers of cells from the surface of the scar. Photographs of stages in the formation of wound gum under these conditions are shown in plates 1, 2, and 3.

Wound-gum zone of the leaf scar						
Reaction of the cell walls of:				Reaction of the material plugging the:		Inter-cellular material
'ortex nchyma	Cortex fiber cells	Xylem parenchyma	Vessels	Lumina of vessels	Intercellular spaces	
ow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
ow, later own	Yellow, later brown	Yellow, later brown	Yellow, later brown	Yellow, later brown	Yellow, later brown	Yellow, later brown
yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow
	Blue	Blue	Blue	Blue	Dark blue	Dark blue
yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow	Deep yellow
	Very light red	—	Light red	—	—	—
	Red	Red	Red	Red	Red	Red
	Blue	Blue	Blue	Blue	Blue	Blue
	—	—	—	Removed†	Removed	Removed
	—	—	—	Removed	Removed	Removed
	Blue	Blue	Blue	Removed	Removed	Removed
t red	Light red	Red	Light red	Removed	Removed	Red
	—	—	—	Removed	Removed	—
	Blue	Blue	Blue	Removed	Removed	—
tropic	Anisotropic	Anisotropic	Anisotropic	Isotropic	Isotropic	Isotropic

In those scars outside moist chambers wound gum usually began to stain first in the walls of the cortex cells, though in many sections the material was noted in the walls of living cells of the xylem in the region of the wound-gum zone (plate 1, *A*). With an increase in the age of the scars came a corresponding development of the wound-gum zone (tables 3 and 4). The intercellular spaces generally filled about the second day. The plugging of the vessels, which usually began about the third day,

TABLE 3

RESULTS OF STUDIES OF THE HEALING OF LEAF SCARS* THAT WERE FORMED IN MAY AND KEPT OUTSIDE OF MOIST CHAMBERS

Age of leaf scars (days)	Presence of wound-gum layer	Depth of wound-gum layer (number of cells from scar surface)	Plugging of vessels by wound-gum		Amount of starch†			Number of scars developing periderm	Number of cell divisions in periderm	Suberinlike material in cells of the wound-gum zone	Scars having oil in cells of wound-gum zone	Tanning in leaf-scar tissues
			Vessels counted	Vessels plugged	Exterior to wound-gum zone	In wound-gum zone	Interior to wound-gum zone					
0	–‡	–	–	–	+++	+++	+++	–	–	–	–	–
1	T	5 to 11	–	–	+++	+++	+++	–	–	–	–	–
2	+	4 to 12	–	–	+++	++	+++	–	–	–	–	–
3	+–	4 to 12	243	24	+++	+–	+++	–	–	T	–	–
4	++	2 to 12	254	70	+++	+	++	–	–	+	1	–
5	++–	4 to 12	264	204	+++	T	++	–	–	+	–	–
6	+++	4 to 12	–	–	+++	–	+	2	1 to 2	+–	3	–
7	+++	4 to 12	270	269	+++	–	+	16	1 to 2	++	2	–
8	+++	4 to 13	...	all	+++	–	T	20	1 to 4	++	2	–
9	+++	4 to 10	...	all	+++	–	T	10	2 to 4	+++	–	–

* Twenty scars were studied at each day group except the ninth day in which 10 scars were used.
† The amount of starch is on a comparative basis.
‡ Legend: – = negative; + = positive; +–, ++, and +++ = increasing amounts in their respective order; T = trace.

was well advanced by the fifth, as shown in table 3 and plate 2, *A*. By the sixth to seventh days the wound gum layer appeared completed in a number of scars (plate 1, *C*, 2, *C* and *D*), and the formation of a periderm had started in a few scars (plate 1, *D* and table 3).

TABLE 4

RESULTS OF STUDIES OF THE HEALING OF LEAF SCARS THAT WERE FORMED IN SEPTEMBER

Leaf-scar number	Age of leaf scar (days)	Wound gum by phloroglucinol in HCl	Orcinol test for wound gum	Depth of wound-gum layer (number of cells from scar surface)	Presence of yellow amorphous material	Copper acetate and ferric chloride test for tannins
			Scars left outside moist chambers			
1	0	—*	—*	—*	—*	—*
2	0	—	—	—	—	—
3	0	—	—	—	—	—
4	0	—	—	—	—	—
5	0	—	—	—	—	—
6	0	—	—	—	—	—
7	0	—	—	—	—	—
8	0	—	—	—	—	—
9	0	—	—	—	—	—
1	1	T	T	5 to 8	+	—
2	1	T	T	7 to 10	+	—
3	1	T	T	8 to 11	+	—
4	1	—	—	—	+	—
5	1	T	T	7 to 9	+	—
6	1	T	T	5 to 8	+	—
7	1	T	T	6 to 10	+	—
8	1	T	T	4 to 8	+	—
9	1	T	T	5 to 10	+	—
10	1	T	T	6 to 11	+	—
11*	1	T	T	6 to 9	+	—
12	1	—	—	—	+	—
13	1	T	T	5 to 8	+	—
14	1	T	T	6 to 11	+	—
15	1	T	T	8 to 12	+	—
16	1	T	T	4 to 9	+	—
17	1	—	—	—	+	—
18	1	—	—	—	+	—
19	1	T	T	7 to 9	+	—
20	1	T	T	9 to 11	+	—
1	2	+	+	4 to 9	+	—
2	2	+	+	5 to 7	+	—
3	2	+	+	6 to 9	—	—
4	2	+	+	7 to 10	—	—
5	2	+	+	4 to 8	—	—
6	2	+	+	6 to 9	+	—
7	2	+	+	6 to 10	+	—
8	2	+	+	7 to 9	+	—
9	2	+	+	6 to 8	+	—
10	2	+	+	4 to 9	+	—

* Legend: — = negative; + = positive; +—, ++, +++ = increasing amounts in their respective order; T = trace.

TABLE 4—(*Concluded*)

Leaf-scar number	Age of leaf scar (days)	Wound gum by phloroglucinol in HCl	Orcinol test for wound gum	Depth of wound-gum layer (number of cells from scar surface)	Presence of yellow amorphous material	Copper acetate and ferric chloride test for tannins
Scars kept in moist chambers						
1	0	–*	–*	–*	+*	–*
2	0	–	–	–	–	–
3	0	–	–	–	+	–
4	0	–	–	–	–	–
5	0	–	–	–	–	–
6	0	–	–	–	–	–
7	0	–	–	–	–	–
8	0	–	–	–	–	–
9	0	–	–	–	–	–
10	0	–	–	–	–	–
1	1	–	–	–	+	–
2	1	T	–	1 to 3	+	–
3	1	–	–	–	+	–
4	1	–	–	–	+	–
5	1	–	–	–	+	–
6	1	–	–	–	+	–
7	1	–	–	–	+	–
8	1	–	–	–	+	–
9	1	–	–	–	+	–
10	1	–	–	–	+	–
11	1	–	–	–	+	–
12	1	–	–	–	+	–
13	1	–	–	–	+	–
14	1	–	–	–	+	–
1	2	T	T	1 to 3	+	–
2	2	+	+	1 to 4	+	–
3	2	+	+	1 to 4	+	–
4	2	+	+	1 to 2	+	–
5	2	+	+	1 to 5	+	–
6	2	+	+	1 to 2	+	–
7	2	T	T	1 to 3	+	–
8	2	+	+	2 to 5	+	–
9	2	+	+	3 to 6	+	–
10	2	T	T	2 to 4	+	–

* Legend: – = negative; + = positive; +–, ++, +++ = increasing amounts in their respective order; T = trace.

Although transverse sections through the wound-gum zone were not very satisfactory for these studies, they show how the wound-gum plugs appear in transverse sections of the vessels (plate 3, *F*).

Two layers of wound gum formed in a number of the scars kept in moist chambers. The first layer usually began to develop in scars collected the second day. Wound gum formed in 1 to 6 rows of cells across the surface of the scar (tables 4 and 5 and plate 3, *A* and *B*) and had generally formed completely by the third to fourth day (plate 3, *C*). About

TABLE 5

RESULTS OF STUDIES OF THE HEALING OF LEAF SCARS* THAT WERE FORMED IN MAY AND PLACED IN MOIST CHAMBERS UNTIL COLLECTED

Age of leaf scar (days)	Presence of wound gum	Number of scars develop-ing two layers of wound gum	Depth of wound-gum layer. (Number of cells from scar surface)		Plugging of vessels by wound gum		Amount of starch†			Number of scars develop-ing periderm	Number of cell divisions	Suberinlike material in cells of the wound-gum zone	Scars having oil in cells of wound-gum zone	Tannins in leaf-scar tissues
			Layer 1	Layer 2	Vessels counted	Vessels plugged	Exterior to wound gum zone	In wound-gum zone	Interior to wound gum zone					
0	–‡	–	–	–	–	–	+++	+++	+++	–	–	–	–	–
1	–	–	–	–	–	–	+++	+++	+++	–	–	–	3	–
2	T	–	1 to 6	–	173	3	+++	+++	+++	–	–	–	–	–
3	+	–	1 to 8	–	203	47	+++	++	+++	1	1	–	1	–
4	+–	3	1 to 5	4 to 10	212	163	+++	+	++	3	1	T	3	–
5	+++	2	1 to 4	4 to 10	...	all	+++	T	+	2	1 to 2	+	1	–
6	+++	13	1 to 4	4 to 8	...	all	+++	T	+	2	1	+–	–	–
7	+++	16	1 to 4	4 to 8	...	all	+++	–	T	3	1 to 2	++	–	–
8	+++	10	1 to 3	4 to 9	...	...	+++	–	T	1	1	+++	–	–

* Twenty scars were studied at each day group except the eighth day in which 10 scars were used.

† The amount of starch is on a comparative basis.

‡ Legend: – = negative; + = positive; +–, ++, +++ = increasing amounts in their respective order; T = trace.

this time a second layer of wound gum began to appear in a layer of cells from 4 to 10 cells from the surface of the scar, below the first layer (plate 3, *D*). Both layers, however, generally converged at the edges of the scar (plate 3, *E*). By the end of the fifth day, wound gum had plugged all the vessels observed in either the first or second zone.

The striking differences in wound-gum formation between the scars left outside moist chambers and those kept in moist chambers are as follows: (1) The scars left outside moist chambers developed only one wound-gum zone, whereas those kept inside developed two layers. (2) The wound gum was usually detectable by the first day in the scars left outside, but not until the second day in those kept in moist chambers. (3) After it once started, however, wound gum formed much more rapidly in the scars kept inside than in those left outside.

Water-Soluble Gum.—The term is used here to designate gumlike materials that are soluble in water as contrasted with wound gum that is insoluble in water. Water-soluble gum, lignin, and wound gum stain blue when treated with orcinol followed by hydrochloric acid. To distinguish, therefore, between wound gum, lignin, and water-soluble gums, a section was first treated with orcinol and HCl. Next, if positive results were obtained, adjacent sections from the same scar were washed in warm water to remove gums, then treated with orcinol. If the material, other than lignified tissues, staining with orcinol was removed by the water, it was considered to be water-soluble gum. Fresh, unfixed leaf scars were used to determine the presence of this material. These studies were made in scars formed September 9. The results for scars kept in moist chambers are recorded under orcinol in table 4, as are also those for scars exposed to outside conditions. Conceivably, water-soluble gums might be deposited in the cell walls before wound gum appeared; but apparently such was not the case, for no positive tests were obtained.

Lignin.—In all cases, phloroglucinol in hydrochloric acid and the Maule reactions were used to distinguish between wound gum and lignin. The former reacts with both substances, whereas the Maule reaction is positive only when the tissues are lignified.

Lee (4) described the abscission of leaves and the healing of leaf scars in numerous species of plants. As to the healing processes of *Castanea sativa* Mill., he states: "Directly after leaf fall—the activity of the cells below the surface of separation is at once shown by the change in the chemical composition of the cell wall. Very gradually these become more or less completely lignified." He does not mention wound gum, but the lignification he describes is probably wound gum, which gives the same reactions as lignin with a number of microchemical tests. If he had used

the Maule reaction, possibly he would have failed to get a positive reaction.

According to results of the present studies of olive leaf scars the vessels, fibers, and a few stone cells were the only tissues of the olive leaf scar that were lignified.

Suberin and Oil.—Sudan III was used to determine the presence of suberin and oil.

In describing the healing of leaf scars of *Castanea sativa,* Lee (*4*) states that when the cell walls of the protective layer undergo "lignification" there is deposited on the inside surface of each cell wall of this layer a fine film of suberin. Lee termed the process "lignosuberization." The protective layer he mentions in the leaf-scar tissue of *C. sativa,* is in a position similar to that of the wound-gum zone in the olive leaf scar.

In the cells of the wound-gum zone of the olive leaf scar was found a material deposited as a thin layer or lamella on the inside surface of the cell walls. This material stained red with Sudan III or IV, was insoluble in alcohol, ether, or benzene, but was soluble in 3 per cent KOH and was isotropic when examined with polarized light. As it did not separate from the cell wall with the protoplasm when the cells were plasmolyzed, it is designated as "suberinlike" material because it apparently has the properties of suberin; but its location in the cell differs from that of suberin, which usually impregnates the wall.

The lamella of suberinlike material began to form after some wound gum had been deposited, and showed first in the 3-day-old leaf scars outside moist chambers (table 3) and in the 4-day-old leaf scars kept in moist chambers (table 5). It continued to develop as the age of the scars increased and stopped about the time the periderm began to form.

Oil globules were found, but not consistently, in either those scars kept in moist chambers or those left outside.

Starch.—Iodine in potassium iodide solution was used for starch determination. In all cases the amount of starch in the cells of the wound-gum zone decreased with increase in the quantity of wound gum (tables 3 and 5).

Tannins.—Sections, after treating with a solution of cupric acetate to precipitate tannins, were rinsed in water and treated with ferric chloride. Tannins stain green, blue, or black. By this method, no difference was noted in the quantity of tannin distributed over the leaf scar at any time during healing.

Periderm Formation.—Observations on the formation of a periderm in the leaf-scar tissue were made when the other healing processes were studied. The results appear in table 3 for the scars left outside moist

chambers and in table 5 for those kept in moist chambers. The periderm developed in the tissue just beneath the wound-gum zone. In the scars left outside, the periderm development was regular. The first cell divisions were observed in two 6-day-old scars. The number of scars in which a periderm had begun to develop increased in each day's collection thereafter. By the eighth day all the scars that were sectioned had completed from 1 to 4 cell divisions in the formation of a periderm (table 3).

The formation of a periderm was not regular in the scars kept in moist chambers. One of the 3-day-old scars, and only one, two, or three of the scars collected each day thereafter, through the eighth day, had started to develop a periderm (table 5). This situation differs from the regular increase in the number of scars that formed periderm in those left outside moist chambers.

From the microchemical studies, one cannot easily explain why the number of scars infected dropped so rapidly during the first two days after the leaves were removed (table 1, and figs. 4 and 5). By the end of *two days there were apparently no materials deposited within the tissues of the scars that mechanically blocked them to invasion by bacteria. After* two days, however, the deposition of wound gum was concurrent with the decrease in the percentage of scars infected. By the time all the vessels of the scars appeared completely plugged with wound gum, the scars had become immune to infection. This held true both for scars kept in moist chambers and for those left outside.

These studies show that during the first two days other factors may be involved in the resistance to infection.

COURSE OF BACTERIA THROUGH LEAF-SCAR TISSUE TO ESTABLISH INFECTION

The processes of healing in the leaf scars previously described do not explain why the susceptibility of scars to infection decreased rapidly during the first two days. They also do not explain why this drop was much more pronounced in the scars kept in moist chambers than in those left outside. A study was made, therefore, of the channels through which the bacteria could enter the tissues to cause infection.

For these studies, a mixture of 1 part of Higgins' American India ink and 5 parts of water proved most satisfactory because it did not diffuse into the living cells and was insoluble in water after it had dried in the scars. This procedure facilitated handling of the scars in water during and after sectioning.

In the experiment several scars were formed as previously described

under methods. One group was placed under moist chambers; another was left outside. When the leaves were being removed and at intervals thereafter, a few scars in each group were covered with the ink mixture, applied with a small brush. The ink mixture usually remained on the surface of the scar about 10 minutes before drying. After the ink dried, the scars were removed and brought into the laboratory for sectioning. The sections were mounted in water, and the depth of ink penetration was measured with a filar micrometer. The greatest depth of penetration

TABLE 6

THE PENETRATION OF INDIA INK INTO THE VESSELS OF LEAF SCARS

Age of leaf scar at time of treatment (hours)	Scars outside moist chambers			Scars inside moist chambers		
	Number of scars treated	Scars in which ink penetrated 5 cells or more (per cent)	Average depth of ink penetration (microns)	Number of scars treated	Scars in which ink penetrated 5 cells or more (per cent)	Average depth of ink penetration (microns)
0	34	86.3	493.3	34	86.3	493.3
½	30	74.2	154.2	20	70.0	139.4
3	25	70.0	65.3	22	63.5	69.5
12	29	48.3	23.9	25	32.0	20.3
24	30	48.2	10.7	22	27.6	8.2
48	25	27.9	8.0	20	15.0	7.3

in each scar was recorded, and the average for each collection was computed from these measurements (table 6, fig. 6). The results show that immediately after the leaves were removed, the ink placed over the scars moves a relatively great distance back into the vessels of the scars. In scars only half an hour old, however, the ink penetrated only a relatively short distance; and this distance decreased thereafter with increasing age of the scar (table 6, fig. 6, *A*). A comparison of these results with those of infection data (fig. 5) suggests that the reason a high percentage of the scars developed knots when inoculated immediately after removal of the leaves was that the inoculum penetrated deeply into the vessels at this time.

In these observations the ink penetrated only through the vessels; in no case was it found to have entered the intercellular spaces or sieve tubes.

If the formation of wound gum in the leaf-scar tissues prevents the entrance of bacteria, the latter, in order to infect the host, must pass beyond the wound-gum zone before it plugs the channels of entrance. The wound gum developed in 4 to 12 cell layers from the surface of the scar. An average thickness of this zone was around 2 to 5 layers of cells.

Apparently, then, if the initial penetration of the bacteria into the scar tissues was 5 cell-layers or more, they were deep enough in the scar tissues to establish infection.

In these studies of ink penetration, therefore, if the India ink placed over the surface of the scar penetrated the tissues 5 cells or more, it was considered deep enough for bacteria to have caused infection. If this

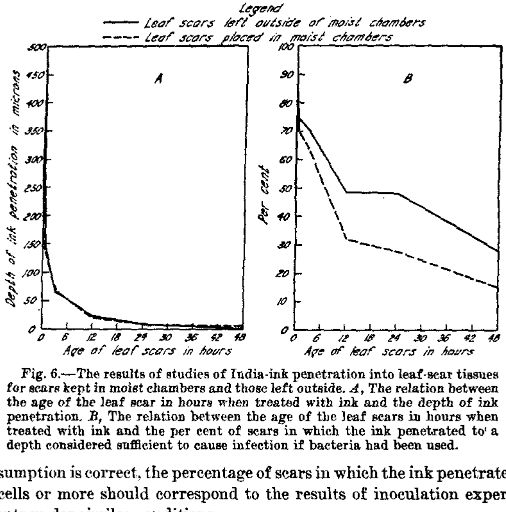

Fig. 6.—The results of studies of India-ink penetration into leaf-scar tissues for scars kept in moist chambers and those left outside. *A*, The relation between the age of the leaf scar in hours when treated with ink and the depth of ink penetration. *B*, The relation between the age of the leaf scars in hours when treated with ink and the per cent of scars in which the ink penetrated to a depth considered sufficient to cause infection if bacteria had been used.

assumption is correct, the percentage of scars in which the ink penetrated 5 cells or more should correspond to the results of inoculation experiments under similar conditions.

The curve (fig. 6, *B*) representing the percentage of scars in which ink penetrated to a depth of 5 cells, or more, compares very closely for the period of the first two days with the results of interval inoculations in the curves of figure 5. For the scars left outside moist chambers the ink penetrated to a depth of 5 cells, or more, in 48 per cent of the scars one day old, and in 28 per cent of those two days old, whereas in the inoculation experiments about 58 per cent of the one-day-old scars developed knots and about 45 per cent of the two-day-old scars developed knots.

For the scars kept in moist chambers the ink penetrated 5 cells or more in about 27 per cent of the one-day-old scars and 15 per cent of the two-

day-old scars. In the inoculation experiments about 23 per cent of the one-day-old scars developed knots, and about 15 per cent of those two days old.

Judging from the ink-penetration studies under the conditions of these experiments, the infection of leaf scars may be influenced by the depth of penetration of the inoculum into the vessels.

THE DEVELOPMENT OF BACTERIA IN THE SCAR TISSUES

The course the bacteria take in establishing infection was studied in artificially inoculated leaf scars. A large number of scars were formed, marked, and inoculated as previously described. Groups of 10 scars each were collected 2, 4, 6, 8, 10, 13, 15, 19, and 26 days after the scars were inoculated. The scar tissues were imbedded in paraffin, as described earlier, and were sectioned with a rotary microtome. The sections were stained with Stoughton's stain for bacteria in tissues (5).

Theoretically the bacteria might infect a leaf scar in several ways: (1) by growth on the surface of the scar, (2) by entering the scar by breaking down the tissue, (3) by penetrating the scar tissue through the intercellular spaces, (4) by entering through fissures in the scar, (5) by entering the scar through the sieve tubes, and (6) by entering the scar tissues through vessels. In the scars examined, the only way, with one exception, in which infection had been established was through the vessels. In one case, however, the bacteria had penetrated deeper than the wound-gum zone through a fissure between cortex parenchyma cells. A photograph of this section is shown in plate 4,*A*. In most cases, according to observations, the bacteria established on the surface, in fissures, and in the intercellular spaces of the leaf-scar tissue are later barred from entering more deeply into the tissue by the formation of wound gum and finally of a periderm.

Bacteria pass deeply into the vessels when the leaf scars are inoculated immediately after the leaves were removed. This point is supported by the experiment in which India ink was placed on the surface of scars and passed freely through the vessels of the leaf and, in a few cases, as deep as the vascular system of the stem. Small groups of bacteria were, furthermore, found scattered along in the vessels (plate 4, *B*) and lodged on the rims at the ends of the vessel cells in sections of scars collected 4 days after inoculation. Bacteria were observed between the secondary thickenings, where they may have lodged as the inoculum was drawn back through the vessels. The groups of bacteria shown in these photomicrographs (plate 4, *B*) are stained very heavily in order that the vessel walls

may be shown. They appear, therefore, only as dark masses lodged between the thickening on the sides of the vessels. These small groups of bacteria enlarge rapidly, producing colonies that finally merge and completely fill the vessels (plate 4, *C*).

The bacteria are freed from the vessels into the other tissues of the leaf scar when the forming periderm pulls the vessels apart (plate 4, *D* and *E*). The periderm forms just beneath the wound-gum zone, and the increase in number and size of the cells pulls the vessels apart at this point. This generally occurs from 8 to 10 days after the scars are formed. The bacteria are then released into the newly forming periderm, a region of actively dividing cells (plate 4, *D* and *E*). The bacteria confined in the vessels deeper in the leaf traces remained there and did not break out into the surrounding tissue by the end of the 19 days after inoculation (plate 4, *E*).

The presence of bacteria or their products among the meristematic cells of the periderm apparently stimulates these cells to active division, and it appears that the successive rows of parenchyma cells were derived from the phellogen (plate 5, *A*). The bacteria increase in numbers at the end of the broken vessels and form small pockets, which increase in size and grow with the surrounding tissue. After 19 days the pockets are fairly large (plate 5, *A*). Some of the cells adjacent to them break down, collapse, and remain around the outer portions of the pockets (plate 5, *B*). As the plate shows, more cell divisions occur in the region around the bacterial pockets.

SUMMARY

A study has been made of certain factors involved in the infection of olive leaf scars by *Bacterium savastanoi* E.F.S. from the stage just before leaf fall until a periderm develops in the scar.

It was found that natural infection in the region of the abscission zone rarely occurs before leaf fall.

Evidence obtained confirms the conclusions of previous workers that most of the new knots forming each year develop at leaf scars.

Most leaf scars were susceptible to infection immediately after leaf fall; the susceptibility dropped rapidly during the first day; and the scars became immune by the end of the ninth. The drop in susceptibility was much more rapid for scars kept in moist chambers than for scars left outside.

Microchemical studies of the abscission processes of leaves and the healing of leaf scars showed that (a) no protective layers are formed in the tissues before leaf fall; (b) separation takes place through the inter-

cellular material between two rows of cells in the base of the abscission zone; (c) the sieve tubes, vessels, cuticle, fibers, and apparently the epidermal cells are mechanically broken at leaf fall; (d) during the healing processes of the olive-leaf scar a wound-gum layer is first formed and is followed by the development of a periderm; (e) water-soluble gums, lignin, oil, suberin, starch, and tannins apparently have no influence upon infection.

India ink was used to trace the course of the inoculum from the surface of the leaf scar into the scar tissues. Judging from these studies, infection may depend upon the depth of penetration of the inoculum.

Most infections in the leaf scars were caused by bacteria that entered the tissues through vessels. Bacteria entering the leaf-scar tissue through intercellular spaces progressed slowly and were stopped by wound gum that plugged these spaces. Those entering the vessels were freed into the periderm cells when the vessels were slowly pulled apart by growth of the periderm. Pockets of bacteria were formed in the tissues derived from the phellogen, and the greatest amount of cell proliferation occurred around the pockets of bacteria.

ACKNOWLEDGMENTS

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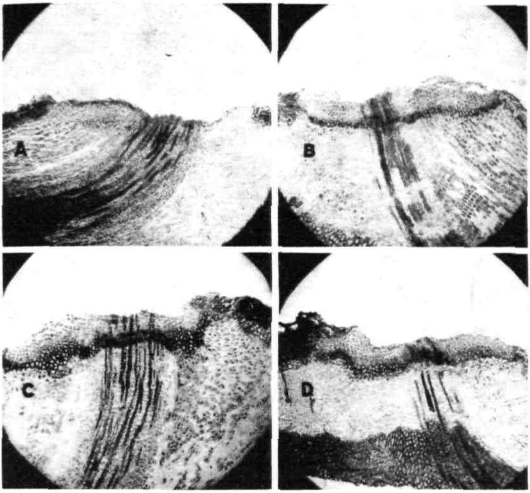

Plate 1.—Sections of leaf scars, approximately 15 microns thick. The sections were treated with phloroglucinol in hydrochloric acid and mounted in the same solution. The lignified walls, a portion of the cell contents, and the wound gum were the only parts of the sections that stained with the phloroglucinol in hydrochloric acid; they appear particularly dark in the photographs. Stages in the development of wound gum in leaf scars left exposed to outside atmospheric conditions: *A*, Longitudinal section of leaf scar 1 day old. The wound-gum layer has begun to form at a distance of 5 to 8 cells from the edge of the leaf scar, as indicated by darker staining in the cortex. *B*, Longitudinal section of a leaf scar 5 days old. The section shows the wound-gum layer being formed and wound-gum plugging some of the vessels. Photographs at higher magnifications showing the plugging of the vessels are in plate 2, *A* and *B*. *C*, Longitudinal section of leaf scar 7 days old. All the vessels of the wound-gum area are completely plugged with gum. Photographs of higher magnifications of the wound-gum zone are shown in plate 2, *C* and *D*. *D*, Longitudinal sections of leaf scars 9 days old. From 1 to 3 cell divisions have occurred beneath the wound-gum zone in an early stage in periderm formation. Also note the vessels that have been pulled apart. (All × 36.)

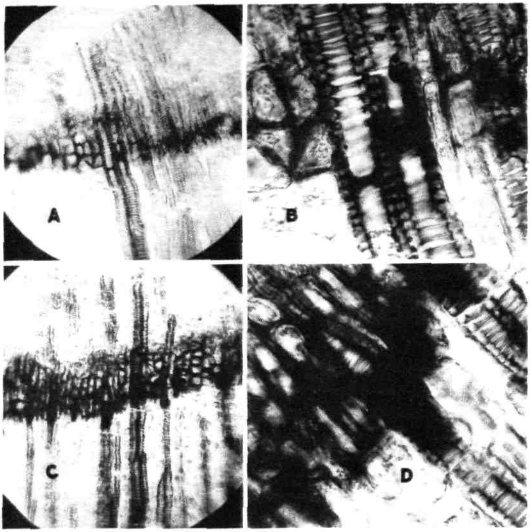

Plate 2.—Vessels plugged by wound gum, and wound gum in the walls of the parenchyma cells: *A*, From the same longitudinal section of a leaf scar as photograph *B* in plate 1. *B*, A portion of the same section as *A*. This shows more clearly the wound gum in the vessels and walls of parenchyma cells. *C*, From the same longitudinal section of leaf scar as photograph *C* in plate 1. *D*, A portion of the same section as *C*. (*A* and *C*, × 192; *B* and *D*, × 694.)

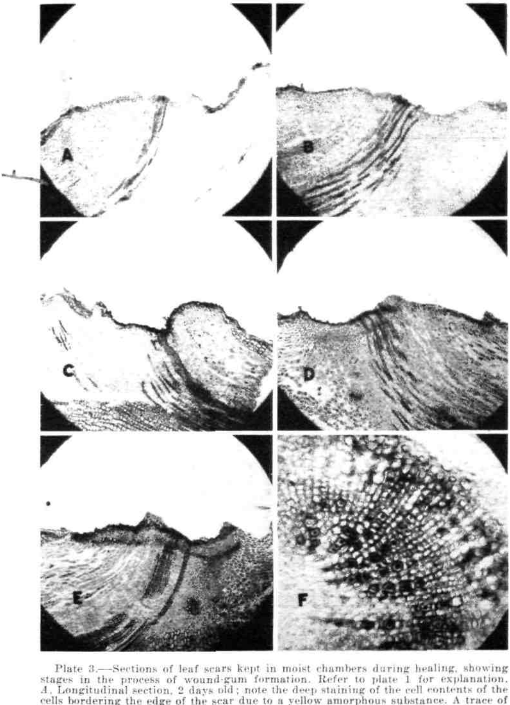

Plate 3.—Sections of leaf scars kept in moist chambers during healing, showing stages in the process of wound-gum formation. Refer to plate 1 for explanation. *A*, Longitudinal section, 2 days old; note the deep staining of the cell contents of the cells bordering the edge of the scar due to a yellow amorphous substance. A trace of wound gum has developed in the upper right edge of the scar. *B*, Longitudinal section, 2 days old, showing a trace of wound gum along the left side in the cells bordering the edge of the scar. *C*, Longitudinal section, 4 days old. The wound-gum layer borders the enire edge of the leaf scar and has completely plugged all vessels and intercellular spaces. *D*, Longitudinal section, 7 days old. The second layer of wound gum has begun to form 3–5 cells interior to the first wound-gum layer. *E*, Longitudinal section, 8 days old. The second layer of wound gum is formed completely. *F*, A portion of a transverse section through the wound-gum zone of a leaf scar 7 days old, showing wound gum plugging some of the vessels. (All × 36.)

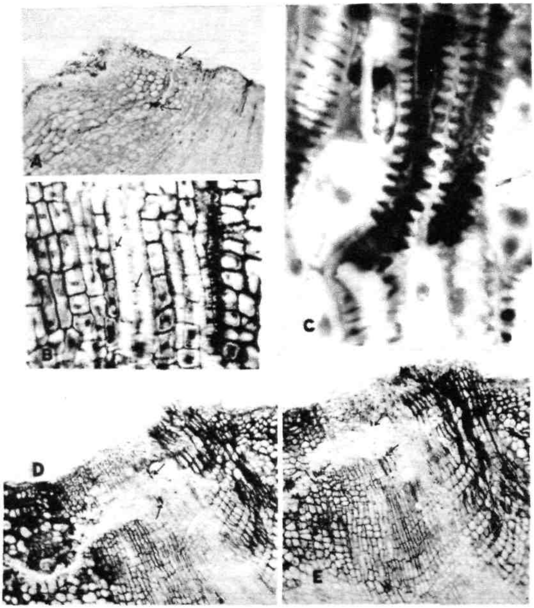

Plate 4.—*A*, Longitudinal section of a leaf scar 12 days old. The leaf scar was inoculated immediately after the leaf was removed. The upper arrow indicates a fissure between the cortex cells through which infection took place, and the lower arrow points to a colony of bacteria. *B*, Vessels from a longitudinal section of leaf scar 4 days after inoculation. The vessels contain colonies of bacteria between the secondary thickenings of the wall, as indicated by arrows. *C*, Vessels from a longitudinal section of a leaf scar collected 7 days after inoculation. They are partly filled with bacterial colonies. The portion of the vessels shown are located just below the wound-gum zone. *D*, *E*, Longitudinal sections of leaf scars collected 12 days after inoculation. Note portions of vessels filled with bacteria and pulled apart and separated by the active division of the periderm cells. The walls from the cells derived from the periderm are not clearly stained. (*A*, × 74; *B*, × 304; *C*, × 1004; *D* and *E*, × 109.)

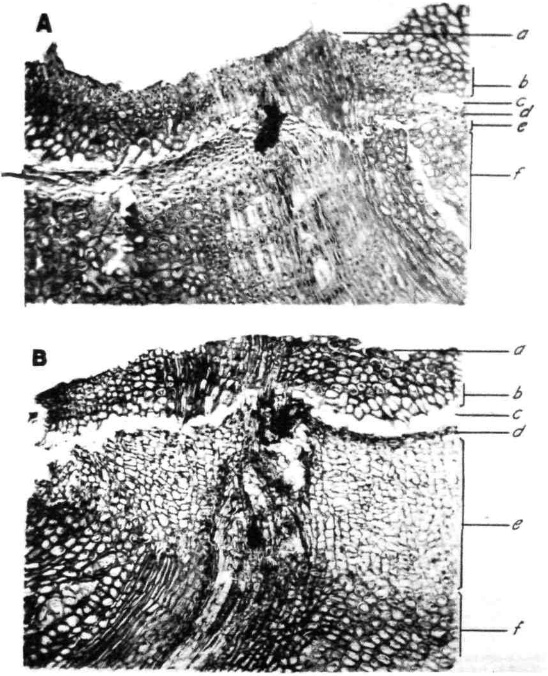

Plate 5.—*A*, Longitudinal section of a leaf scar collected 16 days after inoculation. Note the pocket of bacteria formed among the cells of the periderm. The bacteria in the upper portion of the pocket are extended into vessels. *B*, Longitudinal section of a leaf-scar collected 26 days after inoculation, showing large pockets of bacteria which have developed in the tissue apparently derived from the phellogen. Details are: *a*, leaf-scar surface; *b*, region of the wound-gum zone; *c*, phellem; *d*, phellogen; *e*, tissue derived from the phellogen; *f*, leaf petiole tissue. (All × 133.)

CHARCOAL ROT OF SUGAR BEET

C. M. TOMPKINS

CHARCOAL ROT OF SUGAR BEET[1,2]

C. M. TOMPKINS[3]

INTRODUCTION

CHARCOAL ROT OF SUGAR BEET (*Beta vulgaris* L.), caused by *Macrophomina phaseoli* (Maubl.) Ashby, was found in August, 1932, in Sutter County in the Sacramento Valley and subsequently near Davis, Marysville, Walnut Grove, and on Victoria Island in the delta region west of Stockton. The incidence of infection in numerous fields ranged from 8 to 20 per cent (*5*).[4] Field observations indicated that the fungus attacks half-grown and mature sugar beets during the season of prevailing high temperatures and is probably confined to the hot, interior valleys. It is not known to cause damping-off of sugar-beet seedlings. Inspection of sugar-beet plantings in the cool, coastal valleys has shown them to be free from infection.

In studies of a seedling blight of beans (*Phaseolus vulgaris* L.) caused by this fungus, Kendrick (*3*) showed that the disease was favored by high temperatures. Later, under controlled conditions, Tompkins and Gardner (*6*) corroborated Kendrick's results and found that the fungus from charcoal rot of sugar beet grew throughout a temperature range of 12° to 37° C, with an optimum at 31° and was pathogenic to bean seedlings at high temperatures.

A brief discussion on symptoms of the disease, the causal organism, and pathogenicity of the fungus is presented in this paper.

SYMPTOMS OF THE DISEASE

The leaves of diseased plants show pronounced wilting and eventually turn brown and die. Dead leaves remain firmly attached to the crowns. When an infected plant is pulled from the soil, the symptoms of charcoal rot are distinctive enough to readily differentiate it from all other known root rots of sugar beet. Externally, infection is usually confined to the crown region as indicated by brownish-black[5] lesions of irregular size and shape (fig. 1) and with a silvery sheen. On old lesions the periderm is very thin, papery in texture, and loosely attached to the underlying

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[2] The writer is indebted to Dr. M. W. Gardner for advice and assistance.

[3] Assistant Plant Pathologist in the Experiment Station.

[4] Italic numbers in parentheses refer to "Literature Cited" at the end of this paper.

[5] In determination of root discoloration, Ridgway's (*4*) system of color nomenclature has been followed.

tissues. Under slight pressure it cracks and becomes detached from the root, exposing dry, black, carbonaceous masses of sclerotia (fig. 1).

When examined in cross section soon after infection, the outer or advancing part of a lesion is mustard yellow but later the inner or older

Fig. 1.—*Natural infection of sugar beet by Macrophomina phaseoli;* an advanced stage of infection, showing black lesions with a silvery sheen, which completely involve all of the crown and most of the taproot tissues. The thin, papery periderm has been partly ruptured on the crowns, exposing masses of black sclerotia directly beneath.

part changes to buffy citrine. These colors merge irregularly into each other, with no sharp line of separation (fig. 2). Occasionally the infected tissues may be a uniform buffy citrine. The advancing margin of a lesion, next to apparently healthy tissues, is undifferentiated in color from the tissues invaded earlier and has no distinctive dark band such as characterizes the root rot of sugar beet caused by *Phytophthora drechsleri* Tucker (*7*, fig. 2, *C*). After the entire root has become invaded, the tissues

turn in color from buffy citrine to old gold and finally to brownish black. In the late stages of decay, masses of black sclerotia largely displace the periderm and parenchymatous tissues, forming in pockets or cavities of

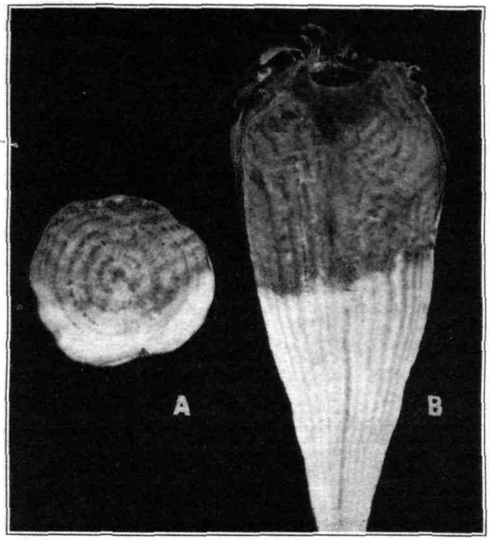

Fig. 2.—Natural infection of sugar beet by *Macrophomina phaseoli: A*, cross section of diseased taproot; *B*, longitudinal section of diseased taproot showing a large mass of black sclerotia at the crown.

irregular size and shape immediately beneath the periderm and extending inward for several centimeters; they may also be found scattered irregularly throughout the mustard-yellow tissues, in marked contrast. Eventually, sclerotial masses occupy the pith and only the vascular elements retain their identity (fig. 3). Completely invaded sugar beets shrink, tend to become mummified, and are of no value for extraction.

Microscopic examination of sections of the infected tissue from inoculated roots stained with magdala red and fast green showed that the mycelium of the fungus was confined to the intercellular spaces.

THE CAUSAL FUNGUS

Tissue fragments from the advancing edge of lesions on approximately 200 sugar beets from various localities were planted on prune agar in petri dishes which were then incubated at room temperature. Colonies containing colorless mycelium usually developed within 24 hours, with but scanty aerial growth. In 36 hours, the medium was darkened by the

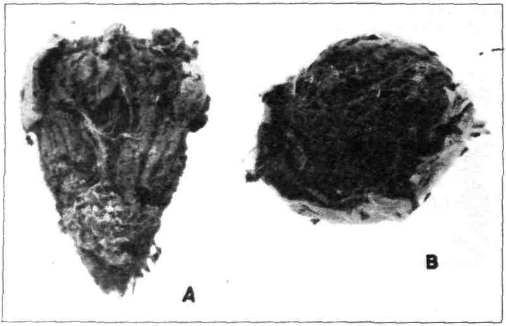

Fig. 3.—Natural infection of sugar beet by *Macrophomina phaseoli*: *A*, longitudinal view and, *B*, top view of crown, showing symptoms typical of the final stages of decay. The periderm has been completely ruptured and, with the parenchymatous tissues, largely destroyed, exposing vascular elements and masses of black sclerotia. Heavy shrinkage and mummification are not uncommon.

formation of sclerotia which in general were small, spherical, and black and rather evenly distributed. Pure cultures were established on prune agar slopes in test tubes by mass transfers of mycelium and sclerotia.

Six isolates of the fungus from diseased sugar beets collected near Stockton, Sutter Basin, and Walnut Grove were grown on potato dextrose agar, pH 5.6, and incubated at 28° for 14 days. A total of 100 sclerotia from each isolate were measured. The diameters of sclerotia ranged from 46.2 to 146.3 microns, with a mean diameter of 73.8 to 87.2 microns. These isolates, therefore, fall within the limits of Haigh's C group, in which the diameter of the sclerotia is 120 microns or less, and, according to the work of Ashby (*1*) and of Haigh (*2*), should be designated as *Macrophomina phaseoli* (Maubl.) Ashby, although no isolate of the fungus from sugar beets has produced any pycnidia.

PATHOGENICITY OF THE FUNGUS

Healthy sugar-beet roots were washed in tap water, rinsed in three changes of sterile distilled water, and allowed to dry. Two small areas on opposite sides from the root sutures were washed with 95 per cent alcohol, after which small cubes of tissue, averaging $^3/_{16}$ inch in size, were removed with a flamed scalpel. Small squares of prune agar containing

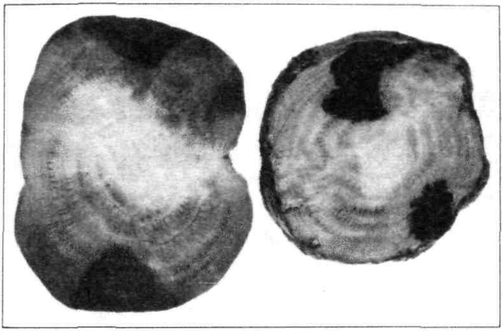

Fig. 4.—Artificial infection of sugar beet by *Macrophomina phaseoli*; cross sections of roots almost completely invaded by the fungus. The black areas on opposite sides represent the sites of inoculation wells and are packed with masses of black sclerotia. A small amount of healthy tissue remains in the centers of the cross sections.

new growth of mycelium and sclerotia were then inserted into these wells, after which the openings were covered with adhesive tape to prevent desiccation. Cultures from Walnut Grove, Sutter Basin, and Stockton, respectively, were tested for pathogenicity. Noninoculated controls received the same treatment except that sterile prune agar was substituted for the inoculum.

After inoculation, all sugar beets were placed in large moist chambers consisting of 5-gallon tin cans with pie tins for covers. Each can was provided with a wire-mesh platform to prevent contact of the roots with the water in the bottom of the can. The inoculated sugar beets were incubated at room temperature (20° to 23° C). After 42 days, 32 roots of 37 inoculated with the Walnut Grove culture, 8 roots of 9 inoculated with the Sutter Basin culture, and 3 roots of 4 inoculated with the Stockton cul-

ture, became infected (fig. 4). The noninoculated controls remained healthy. The fungus was reisolated in pure culture from all diseased sugar beets. The reisolated fungus proved pathogenic upon inoculation into healthy sugar beets.

Six sugar beets were inoculated by placing inoculum on the unwounded periderm under aseptic conditions and were held in moist chambers. After 15 days, 5 roots were infected and the fungus was reisolated. This suggests that the fungus may penetrate the unwounded periderm.

Isolates of the fungus from bean (*Phaseolus vulgaris* L.) var. Red Mexican, tuberous begonia (*Begonia tuberhybrida* Voss), cotton (*Gossypium hirsutum* L.), strawberry (*Fragaria* sp.), and sweet potato (*Ipomoea batatas* Poir.) proved highly pathogenic to sugar-beet roots within 15 days after wound inoculations were made. The symptoms produced by these isolates in sugar-beet roots were identical with those resulting from inoculation with the isolates from sugar beet.

The susceptibility of sugar-beet seedlings to infection was tested in paraffined paper cups containing autoclaved sand to which the fungus was added (*6*). After 7 days, it was observed that the fungus attacked cotyledons as well as the roots and stems of seedlings. At room temperature, only 3 of 56 seedlings inoculated were infected; at 25° C, 3 of 53 seedlings; at 28°, 9 of 31 seedlings; at 31°, 21 of 25 seedlings; and at 34°, 8 of 13 seedlings. Controls at each temperature continued healthy. The fungus was readily isolated from diseased seedlings and again proved pathogenic upon inoculation. These results show that higher temperatures are especially favorable to infection.

SUMMARY

A crown rot of sugar beet, caused by *Macrophomina phaseoli* (Maubl.) Ashby, is described.

The disease occurs only in the interior valleys of California and is apparently dependent upon high temperatures.

Infection of sugar-beet roots and seedlings was obtained in the laboratory with different isolates of the sclerotial form of the fungus from sugar beet.

Infection of sugar-beet roots was also obtained in the laboratory with isolates from other hosts.

The optimum temperature for growth of one of the isolates from sugar beet was shown to be approximately 31° C.

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